Scuola Internazionale Superiore di Studi Avanzati - Trieste

Study of the localization of the Human Papillomavirus E6 and E7 proteins: implications for their oncogenic function.

Thesis submitted for the Degree of Doctor Philosophiae

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SUMMARY

Mucosal Human Papillomaviruses (HPVs) are the causative agents of a number of human pathologies, including benign condylomas, as well as the majority of cervical cancers and their high-grade precursor lesions. Although the viral E6 and E7 proteins are known to be essential for driving malignant progression of HPV infected cells, there are still many uncertainties about their mode of action. In this thesis I have analysed the intracellular distribution of the E6 and E7 oncoproteins from the high-risk HPV-18 and HPV-16, and the low-risk HPV-11 as well as that of the HPV-18 E6*I short peptide.

I first show that the E6*I protein has a regulatory function on the full length E6 protein, rescuing p53 from E6 mediated degradation, and that this function is modulated during the cell cycle, as the respective cellular abundance of the two proteins varies, with specific upregulation of E6*I being detectable during G2/M.

Next, I show that both full length E6 proteins derived from HPV-11 and HPV-18 localise within the nucleus in nuclear bodies that are confocal with the promyelocytic leukaemia (PML) protein. Using a panel of different PML isoforms, I demonstrate specific co-localization between the E6 proteins and PML isoforms I-IV but not with PML isoforms V and VI. I also demonstrate interaction between E6 and a subset of PML isoforms both *in vitro* and *in vivo*. As a consequence of this interaction the insoluble form of PML IV is destabilised by HPV-18 E6 through a proteasome dependent pathway. Interestingly, both HPV-11 E6 and HPV-18 E6 can readily overcome PML IV induced cellular senescence in primary cells. These results show separable functions for different PML isoforms that are specifically targeted by the HPV E6 oncoproteins.

Finally I demonstrate that upon the expression of the ubiquitin ligase E6-AP, it colocalises

together with HPV-18 E6 in the cytoplasm and in PODs containing PML isoforms I-IV. Moreover, a mutant of E6 lacking its Nuclear Localization Signal (NLS), which is mainly localised in the cytoplasm, is still able to enter the nucleus "piggy-back" on E6AP and to localise into PODs. This subset of PODs is enriched in proteasomes and in poly-ubiquitin conjugated proteins, suggesting that they are nuclear sites of proteasomal degradation induced by E6. Indeed, in the presence of E6, p53 levels are decreased and that p53 which is still present co-localises with E6 and E6AP in E6-induced nuclear dots.

These studies provide new insights into the spatial organization of E6 induced proteasomal degradation, and demonstrates how targeting nuclear structures such as PODs, which are highly enriched in specific regulatory proteins, might be essential for low abundant proteins such as E6 and E7, to exert their profound effects upon the cellular homeostasis.

INTRODUCTION

The complexity that characterises the structure of the eukaryotic cell is maintained by a highly compartmentalised environment. At one level, this environment is defined by membranes, that physically separate different organelles such as lysosomes, mitochondria, endosomes, and the Golgi apparatus, from the cytosol, as well as the latter, from the nucleus. At a second level, proteins themselves can aggregate and form a micro-environment enriched in certain enzymes or factors, thus creating a "structure" with a specific function. Examples of these "structures" exist both in the cytosol, such as the aggresomes (Johnston et al., 1998; Kopito, 2000), and in the nucleus (see **Table 1**), thereby further augmenting the level of complexity of eukaryotic cells. Obtaining access to such "structures" is an essential step in the function of key cellular regulatory proteins, which include the two major "guardians" of the cell, the tumour suppressors p53 and pRb. The importance of this intracellular organisation is further attested to by the dysfunctions that correlate with mislocalization of nuclear proteins in human disease and cancer. Determining the subnuclear localization of proteins is therefore important in gaining a full understanding of their regulation and function, as well as to provide clues on the putative function of novel proteins.

From the point of view of a small DNA virus such as Human Papillomavirus (HPV), that encodes only a small number of low-abundant regulatory proteins, targeting certain essential "structures" must be crucial to obtain full use of the cellular machinery in order to fulfil its needs.

In this introduction I will therefore give an overview of some of these nuclear "structures" and on the general features of HPV and HPV encoded proteins, leaving the rest of my

thesis to the aim of bringing these two topics together.

The nucleus

The mammalian cell nucleus stores the genomic information and contains the machinery essential for gene transcription. In interphase nuclei, chromosomes occupy discrete areas referred to as **chromosome territories** (Schardin et al., 1985), which are separated by channels called the interchromosomal domains. Active genes are preferentially localised towards the inner part of the chromosome territories, while **heterochromatin** is observed more towards the periphery of the nucleus, adjacent to the nuclear lamina. The area not occupied by DNA is called the **interchromosomal** region, and these sites are highly enriched in processing and transport factors (Wansink et al., 1996).

As mentioned above, the nucleus has a complex organization, with a variety of discrete domains that have been described in recent years (Lamond and Earnshaw, 1998; Spector, 2001). These domains have been shown to be dynamic structures, with rapid protein exchange occurring between them and the nucleoplasm (Misteli, 2001). In the next section I will try to summarize the most important nuclear domains that have been described, highlighting their specific role and function.

Functional Domains within the Nucleus

An indication of the complex internal organisation of the nucleus comes from the identification of defined regions to which certain nuclear processes are localised. Rather than taking place throughout the nucleus, activities such as DNA replication, splicing and transcription may be localized to discrete sub-nuclear structures or domains. The nature and function of these nuclear structures are becoming more clearly defined and a list of them is

summarized in **Table 1** as well as represented in Fig. 1.

Table 1

	N.	Dimension µm	Function/Role	Proteins/molecules contained
Replication Factories	150-200	0.2-0.5	Cluster of replication origins	PCNA, DNA polymerase alpha
Speckles	20-50	0.8-1.8	Pre-mRNA processing, pre-mRNA splicing factors assembly/modification	SF2/ASF, SC35
OPT	1-3	1.0-1.5	Transcription	Oct1, PTF, TBP, Sp1, RNAPol II
PcG bodies	2-200	0.2-1.5	Associated with pericentromeric heterochromatine: possible role in silencing.	Polycomb group proteins: RING1, BMI1, hPc2
Cajal bodies, Coiled bodies	1-10	0.2-1.0	snRNP biogenesis and in the trafficking of snRNPs and snoRNPs	snRNPs (U1, U2, U4/U6, U5, U7) snoRNPs (U3, U8)
Gems	1-10	0.3-1.0	snRNPs maturation	SMN, Gemin2
Cleavage bodies	1-4	0.3-1.0	mRNA processing	CstF, CPSF
Nucleoli	1-10	0.5-5.0	Ribosome biogenesis: rRNAs transcription, processing and assembly	rRNAs, snoRNPs
Perinucleolar Compartments (PNC)	1-10	0.25-1.0	Not clear: found in cancer cells	Poly Pyrimideine Tract Protein (PTB)
SAM68 bodies	1-10	0.25-1.0	Not clear: found in cancer cells	RNA-Binding proteins containing a GSG/STAR domain (SAM68)
Stress bodies, HAP bodies	1-10	0.25-1.0	Not clear: stress specific splicing; depot of mRNA	HAP, SAM68, SRp30c, SF2/ASF
PML bodies, ND10	5-30	0.3-1.0	Transcription regulation, apoptosis, Antiviral response, Nuclear depot/dump	PML, Sp100, p53, pRb, SUMO-1, Daxx, HDACs, eIF4E, CBP/p300

The nuclei of mammalian cells appear to contain clustered sites of DNA replication within which the replication of multiple DNA molecules takes place. These discrete sites of DNA replication have been defined by experiments in which newly synthesized DNA was visualized within cell nuclei, and allowed the staining of approximately 150-200 discrete clusters distributed throughout the nucleus. These **"Replication Factories"**, appear at the

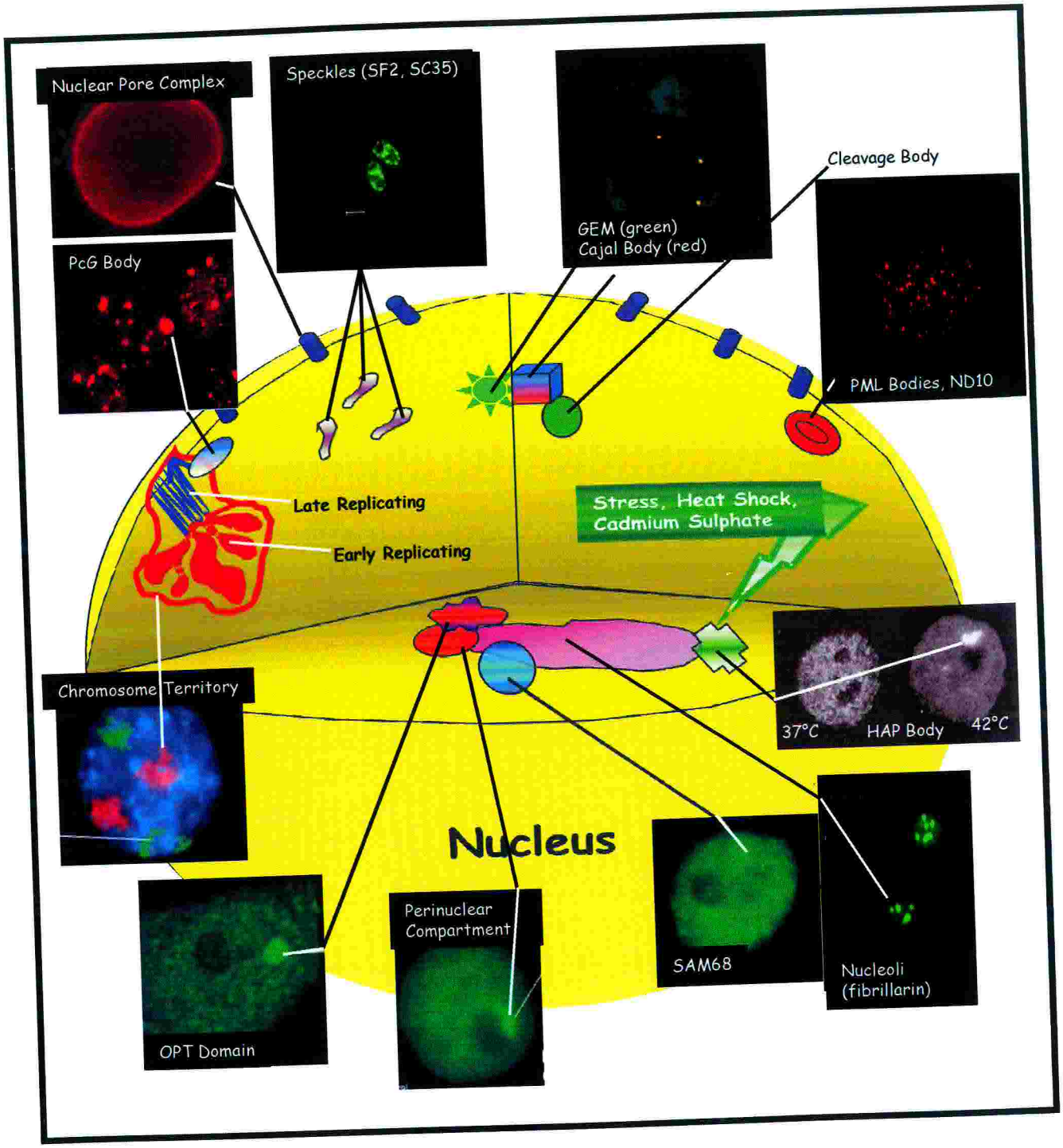


Fig. 1 Schematic representation of the cell nucleus. Nuclear Structures are highlighted, and their appearance under a fluorescent microscope is shown.

end of the G1-phase and quickly become active; as S-phase progresses, they increase in size and decrease in number, because of fusion occurring between smaller foci (Hozak et al., 1993; Hozak et al., 1994). The existence of such domains provided the first evidence for replication occurring at discrete sites attached to the nucleoskeleton. Recently the X-ray structure of a hexameric SV40 Large T Antigen (LTag) with DNA helicase activity was solved, and this revealed that it acts as a double hexamer, similarly to the MiniChromosome Maintainance (MCM) complex (Fletcher et al., 2003). The most interesting finding, however, is that it allows unwinding of the DNA duplex bi-directionally, by means of the so called "iris" mechanism, while ssDNA loops are released from the side channel of the helicase domains (Li et al., 2003). Thus, if we extend the similarity to eukaryotic MCMs, this suggests a model by which replication occurs as the DNA template moves through these fixed domains, rather than moving along the DNA itself. Since approximately 4000 origins of replication are active in a diploid mammalian cell at any given time, each of these clustered sites of DNA replication is expected to contain approximately 20-40 replication forks (Hozak et al., 1994). Thus the so called "replication factories" are the first example of the aforementioned compartmentalization.

A second example of these domains are **Nuclear Speckles**. These are dynamic structures containing pre-messenger RNA (mRNA) splicing factors and other proteins that are involved in transcription and splicing. They vary in number between 20 and 50 and their size and abundance is regulated during the cell cycle, ranging from 20-25 nm in diameter for a single particle, to 0.8-1.8 μm in diameter for clustered structures. These larger aggregates correspond to **Interchromatin Granule Clusters (IGCs)** (Spector, 1993). The role of IGCs is not yet fully understood, however their disassembly perturbs both transcription and pre-mRNA splicing in mammalian cell nuclei suggesting a role in

coordinating these two processes (Sacco-Bubulya and Spector, 2002). The general model is that speckles are storage sites of splicing components, which are then recruited from these sites to actively transcribed genes, where pre-mRNA processing occurs; they do not contain DNA and they are not principal sites of transcription (Lamond and Spector, 2003). This process is regulated by a cell cycle regulated kinase termed SRPK1, which has a specificity for SR (Serine Arginine) splicing factors, and seems to play a crucial role in controlling the intranuclear distribution of splicing factors in interphase cells, and in reorganizing nuclear speckles during mitosis (Gui et al., 1994). This regulation makes speckles highly dynamic structures, with splicing factors cycling continually inwards and outwards.

In addition to speckles, nuclei contain sites of transcription, although these are generally diffused throughout the nucleosol. One exception to this is represented by the so called **OPT domains**. The name stands for Oct1-PTF-Transcription domains and they are found in about 30% of asynchronous cells (Grande et al., 1997). They are enriched in nascent transcripts as well as transcription factors and have a cell cycle dependent distribution, being associated with the nucleoli in G1 and early S phase and disappearing after that (Pombo et al., 1998). Their role is still unclear, although they are thought to act similarly to nucleoli, bringing particular genes present on specific chromosomes to a region enriched in transcription and processing factors, thus facilitating their expression (Pombo et al., 1998).

As described in the previous section, chromosomes occupy specific regions termed chromosome territories, and within these domains there is a spatial organization of highly packed and inactive heterochromatin and less tightly packed and more transcriptionally active euchromatin (Cremer et al., 2000). **PcG bodies,** are a subtype of nuclear bodies, which are associated with etherochromatic regions. The larger PcG foci are generally localized near the centromeres, and they can be visualized with a kinetochore antibody

marker (Saurin et al., 1998). The Polycomb group (PcG) of proteins contained in these structures, are thought to maintain a repressed transcriptional state on chromatin, and can form large multiprotein complexes. Characterisation of the Drosophila M. complex (Saurin et al., 2001) showed that over 30 different proteins are involved in the silencing of essential genes required for development, and that the PcG complex is also directly linked to the basal transcription machinery (Satijn et al., 1997; Saurin et al., 2001). Finally it is worth mentioning that E2F6, a member of the E2F family that does not interact with the pocket proteins, mediates its transcriptional repression activity through this complex (Trimarchi et al., 2001).

The **Nucleoli** are structures formed around the ribosomal DNA (rDNA), which can be specifically found at chromosomal loci called NOR (Nucleolar Organizing Regions). Within the nucleoli, rRNAs are transcribed, processed and assembled (Shaw and Jordan, 1995). The number of nucleoli varies during the cell cycle from between 1 and 10 and its maximum is connected with the expression of rDNA from the 10 NORs on chromosomes 13, 14, 15, 21 and 22 (Shaw and Jordan, 1995). Specifically there is an initial formation of small nucleoli, each around a single NOR, which then tend to fuse into one, by cooperative stimulation during interphase. Dissociation of this big nucleolus occurs upon entering prophase, due to the condensation of the chromosomes and consequent retreat of rDNA. As a result of this, the nucleolar number increases again (Anastassova-Kristeva, 1977). The size of nucleoli ranges from between 0.5-5.0 μm in diameter, and three regions are clearly identifiable: the **fibrillar centers**, the **fibrillar component**, which is the region connecting the fibrillar centers, and where rRNA transcription and processing most likely occur, and finally the **granular region**, which contains pre-ribosomal particles at different stages of maturation, as well as large and small ribosomal subunits (de Carcer and Medina, 1999;

Hyttel et al., 2000; Laurincik et al., 2000).

Three other defined structures in close association with the nucleolus have been characterized: the **perinucleolar compartment (PNC),** the **SAM68 nuclear body** (Huang, 2000) and the **stress-induced nuclear bodies** or **HAP bodies** (Chiodi et al., 2000). The **perinucleolar compartment (PNC)** is a unique nuclear structure containing small RNAs transcribed by RNA polymerase III and the polypyrimidine tract binding protein (PTB; hnRNP I) (Ghetti et al., 1992; Matera et al., 1995; Lee et al., 1996). Although the functions of PNC and SAM68 nuclear bodies are unknown, both of these structures are predominantly found in cancer cells, and they are rarely observed in primary cells (Chen et al., 1999). Moreover increased levels of Sam68 protein can significally increase cell proliferation, further implicating Sam68 function in tumorigenesis (Liu et al., 2000).

Finally **Cajal bodies** (Matera et al., 1994) and **Gems** (gemini of Cajal bodies) (Matera, 1999) are overlapping bodies involved in snRNP (small nuclear Ribo Nucleo Protein) biogenesis and maturation (Matera et al., 1994). Other characterized structures involved in mRNA processing are the so called **Cleavage Bodies**, which, as the name suggests, are mainly involved in the cleavage and polyadenylation steps of mRNA processing (Schul et al., 1996).

There is one specific type of nuclear structure that is of particular interest for the understanding of many cellular pathways, such as the apoptotic response, cell cycle regulation, senescence and cell cycle arrest, transcriptional activation and repression, as well as protein degradation through the proteasome pathway. These structures are the **PML bodies**, also known as **PODs** (PML Oncogenic Domains), **ND10** or **Kr Bodies** (Ascoli and Maul, 1991; Dyck et al., 1994), and because of their relevance in this series of studies, they

will be described in detail below (see **Fig.2**). For clarity I will refer to these structures as **PODs** throughout my thesis. The number of PODs in a normal cell ranges between 5 and 30, depending on the phase of the cell cycle and on external stimuli (Everett et al., 1999), while their size ranges between 0.3 and 1.0 μm. They are intimately associated with the nuclear matrix, although they contain neither chromatin nor nascent RNA (Boisvert et al., 2000), and they are dynamic structures, of which the PML protein is the major regulator, controlling POD organization and function (Dyck et al., 1994; Lallemand-Breitenbach et al., 2001).

PML and APL

PODs are implicated in the pathophysiology of Acute Promyelocytic Leukaemia (APL) (Alcalay et al., 1992; Chen and Chen, 1992). They are specifically disrupted in APL cells (Acute Promyelocytic Leukaemia) and their main component, the PML protein, is fused to the retinoic acid receptor alpha (RARα), due to a reciprocal translocation, to form the oncoprotein PML-RARα (de The et al., 1991); therefore PODs are not present in APL cells, while PML, PML-RARα and the other steroid receptor RXR associate throughout the nucleoplasm in smaller foci (Koken et al., 1994). As well as having a certain nuclear fraction, PML-RARα has also been found to be predominantly localised in the cytoplasm. Retinoic Acid (RA) administration is used to treat APL patients since it induces blast differentiation and consequently complete remission (Kakizuka et al., 1991; Zhu et al., 1997). The molecular mechanism by which this occurs resides in the re-aggregation of PML into the PODs, following RA treatment, while PML-RARα remains mainly cytoplasmic (Daniel et al., 1993). Thus, PML-RARα expression leads to a RA-reversible

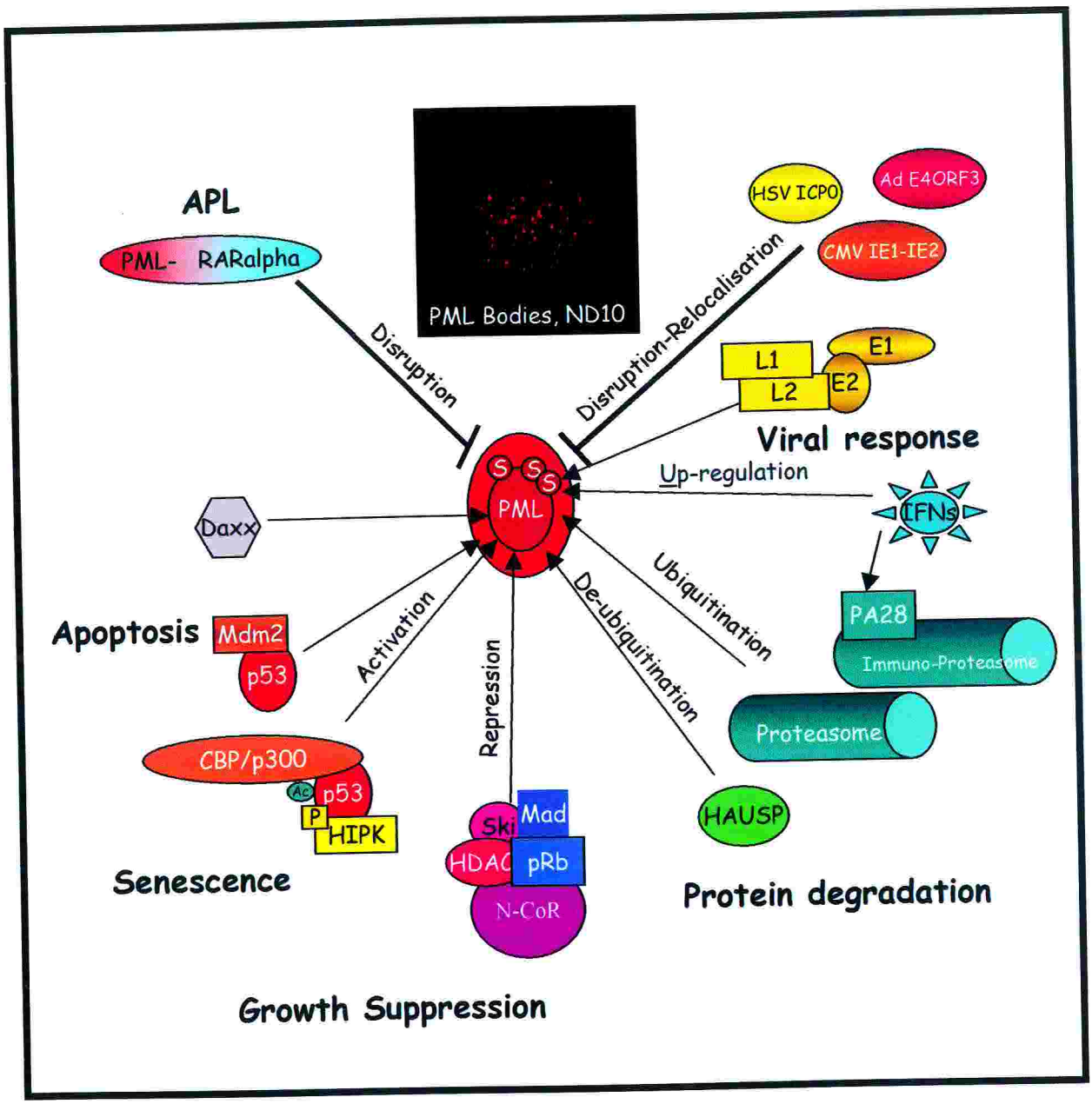


Fig. 2 Role and Function of PML interacting proteins. PML has a central role in regulating apoptosis, senescence, growth suppression, protein degradation and viral response. Abbreviations: APL (Acute Promyelocitic Leukaemia), S (SUMO-1), P (Phosphorylation), Ac (Acetylation).

alteration of a nuclear domain, acting in a dominant negative fashion on the wild type PML protein (Daniel et al., 1993). An alternative drug that is used to treat APL patients is Arsenium Trioxide (As_2O_3), which can induce clinical remission in de novo or relapsed APL patients, and has no cross-resistance with ATRA (All-trans Retinoic Acid). As_2O_3 has a dual effect on APL cells, preferentially inducing apoptosis at high concentration, and partial differentiation at lower doses (Chen et al., 1996; Chen et al., 1997).

Identification of alternatively spliced PML transcripts

The PML gene comprises nine exons that are alternatively spliced and which give rise to at least seven different isoforms (de The et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991; Fagioli et al., 1992; Kastner et al., 1992). These isoforms can be divided into seven groups, PML (I-VII), and they all differ within their C-terminal regions. All of them, with the exception of isoform VII, localise in discrete domains within the nucleus (Jensen et al., 2001). A further sub-grouping can be made due to alternative splicing within exons 4, 5 and 6, and are called a, b and c (de The et al., 1991; Reymond et al., 2001). It is possible that all the above mentioned isoforms can exist as a, b or c forms, although not all the sequences are yet available (Fagioli et al., 1992). One important feature of these different isoforms is that they vary in their localization, since only the "a" form has a functional NLS sequence, encoded by exon 6. Based on this information it is thought that all PML isoforms (I-VI) should exist in both nuclear and cytoplasmic locations, possibly having different functions in each. Finally it should be noted that at a transcriptional level, all the isoforms are expected to be equally expressed (Fagioli et al., 1992).

PML protein domains and post-translational modification

The PML protein has three cysteine rich binding domains, an RBBC motif (Ring finger, B boxes and an alpha helical Coiled-Coil domain)(Borden et al., 1995; Jensen et al., 2001) and can be modified by the small ubiquitin-like protein SUMO-1, at lysines 65, 160, and 490 (Kamitani et al., 1998; Duprez et al., 1999). This modification is essential for correct formation of PODs (Zhong et al., 2000a; Lallemand-Breitenbach et al., 2001) and is thought to be involved in modulating protein-protein interactions. Finally a carboxy terminal coiled-coil region is responsible for heterodimerization between different PML proteins (Jensen et al., 2001). Interestingly, PML SUMOylation is the only SUMO-1 modification found to be essential for POD targeting. Indeed a growing number of proteins that have been shown to be modified by SUMO-1 at the specific ΨKXE consensus (Rodriguez et al., 2001) and to interact with PML, such as p53, DAXX, SP100 and CBP/p300, continue to localise in PODs even in the absence of such modification (Sternsdorf et al., 1997b; Gostissa et al., 1999; Kwek et al., 2001; Jang et al., 2002). Thus the initial theory that SUMOylation was a prerequisite in POD targeting are being revised (Muller et al., 1998). PML is also modified by phosphorylation, both on tyrosine and serine residues (Chang et al., 1995), and it was recently shown that Homeodomain-Interacting Protein Kinase-2 (HIPK-2) is involved in this process (Engelhardt et al., 2003).

The role of PML in the control of protein stability

Among the many different functions that have been attributed to PODs, one of the most recently studied is their possible role in intranuclear proteolysis. PML, as mentioned above is a RING finger protein, and this family of proteins has been implicated in proteasome mediated degradation (Ulrich, 2002). Moreover it shares homologies with COP-1 (Reyes,

2001), a member of the signalosome, involved in protein degradation both in plants and eukaryotes (Mundt et al., 1999; Schwechheimer and Deng, 2000; Suzuki et al., 2002).

This link between PML and the proteasome pathway has not yet been fully verified, but several observations point in this direction. First of all, SUMOylated PML promotes its own catabolism, recruiting to PODs the proteasome machinery, and thereby creating a favourable environment for the accumulation of components of the ubiquitin pathway, and therefore promoting the degradation of other proteins (Anton et al., 1999; Lallemand-Breitenbach et al., 2001). Secondly PODs are also enriched in ubiquitin proteases, such as USP7/HAUSP (Everett et al., 1997). Moreover they have been shown to play a role in IFN induced MHC class I antigen presentation: the mechanism seems to involve the PA28 protein, an IFNγ inducible proteasome activator able to induce the formation of 'immunoproteasomes' at these sites (Fabunmi et al., 2001).

Finally the most interesting observations regarding this function of PML came very recently from the work on PML and another RING finger protein, Mdm2. An initial study suggested that PODs were sites of Mdm2 and p53 accumulation following the inhibition of nuclear export (Lain et al., 1999). Both proteins become relocalised to PODs after LMB (Leptomycin) treatment and the net result is an increase in p53 stability. This finding suggested PML as being a possible negative regulator of Mdm2, and this was indeed subsequently demonstrated in a following study which showed that PML and Mdm2 can directly interact, and that this interaction involves more than one domain (Wei et al., 2003). As a result of this binding, PML protects p53 from Mdm2-mediated ubiquitination and degradation by competing for the same binding region (Zhu et al., 2003), thus enhancing its transcriptional activity (Louria-Hayon et al., 2003b). This seems to be of crucial importance during the stress response, with PML playing a central role in the activation and

stabilisation of p53 in response to cellular stress and DNA damage caused by UV-irradiation (Kurki et al., 2003).

PML role in tumor suppression

As mentioned above, the presence of PML in PODs is essential for the recruitment of other proteins, since in *PML*$^{-/-}$ cells, each PML-NB component so far analysed acquires an aberrant nuclear localization pattern, and is rescued to a normal localization by the reintroduction of PML (Zhong et al., 2000a; Lallemand-Breitenbach et al., 2001). These findings imply a structural role for PML in PODs biogenesis, which may underly the ability of PML to interact with at least some of the cellular proteins that have been shown to target PODs (Jensen et al., 2001). Among these interactions the ones between PML and p53, pRb, DAXX, CBP, HDACs and eIF4E have been extensively characterized (Alcalay et al., 1998; LaMorte et al., 1998; Ishov et al., 1999; Fogal et al., 2000; Lai and Borden, 2000; Wu et al., 2001). Through the interaction with all these key regulators of the cell homeostasis, PML plays a central role in critical tumor suppressive functions such as induction of apoptosis, growth arrest, and cellular senescence.

Earlier studies have indeed shown that PML is a bona fide tumor suppressor, being able to reduce colony formation in rat embyo fibroblasts induced by cooperative oncogenes, to suppress anchorage-independent growth of NB4 cells (an APL derived cell line) in soft agar, and to suppress transformation of NIH 3T3 cells by an activated neu oncogene (Mu et al., 1994). Moreover, overexpression of PML in tumour cells after transduction with a recombinant adenovirus is also able to significantly suppress the growth rate, clonogenicity and tumorigenicity of breast cancer cells (Le et al., 1998), prostate cancer cells (He et al., 1997) and bladder cancer cells (He et al., 2003).

Interestingly cotransfection of PML with PML-RARα results in a significant reduction in PML's tumor suppressor functions, indicating a dominant negative role for the fusion protein in APL cells (Mu et al., 1994). The best evidence of PML tumor suppressive activity *in vivo* came however, when PML knock-out mice were obtained (Wang et al., 1998a). $PML^{-/-}$ MEFs have the ability to grow to higher cell densities, and to form a higher number of colonies and foci, when compared to their wild type counterparts. However they are unable to grow in a semisolid medium, unlike fully transformed cells (Wang et al., 1998a). Interestingly PML-/- mice are viable, but they usually die within a one-year follow-up period due to infections. Although this does not allow for long periods of observation, it is surprising that, within this time frame, they do not spontaneously develop tumours. However, $PML^{-/-}$ mice are highly susceptible to tumour development following several physical/chemical insults, such as DMBA, TPA or sublethal doses of γ-irradiation (Wang et al., 1998a; Wang et al., 1998b). Taken together, these data indicate that PML can act *in vivo* as a tumor suppressor. Moreover, in APL, PML is reduced to hemizygosity as a consequence of the chromosomal translocation t(15;17). This in turn facilitates the dominant-negative action of the PML-RARα fusion protein. Definitive support for this hypothesis was obtained in vivo, by crossing PML-RARα transgenic mice with $PML^{-/-}$ animals. *PML* inactivation resulted in a dramatic increase in leukemia incidence and an acceleration of its onset. Leukemogenesis was also markedly enhanced in a $PML^{+/-}$ background, demonstrating that PML is haploinsufficient in this respect (Rego et al., 2001). A final point that deserves mentioning is the fact that the PML-RARα expressing mice, developed not only leukaemia, but also skin papillomas, implying that PML plays an essential role in controlling skin cell proliferation, and that deregulation of PML and RARα signalling at this site, in the absence of RA production, leads to tumor induction (Hansen et al., 2003).

The role of PML in apoptosis.

Clear evidence for the implication of PML in regulating apoptotic events comes from experiments performed on *PML*$^{-/-}$ mice. These mice display protection from several apoptotic pathways including those induced by FAS, tumor-necrosis factor-α (TNFα), ceramide, and IFN types I and II (Wang et al., 1998b). Moreover *PML*$^{-/-}$ mice and *PML*$^{-/-}$ cells are resistant to the lethal effects of γ-irradiation, suggesting that PML is also important in the process of DNA damage-induced apoptosis (Wang et al., 1998b). The DNA damage response activates checkpoint kinases, that induce cell cycle arrest and DNA damage repair pathways, and in some cells apoptosis. The main response seems to involve p53 stabilization by the chk2 kinase (Hirao et al., 2000). However, γ-irradiation-induced apoptosis also occurs through p53-independent mechanisms (Strasser et al., 1994; Merritt et al., 1997). Thus PML seems to have both p53-dependent and independent roles in apoptosis following γ-irradiation. The p53-dependent pathway seems to be modulated by PML through direct interaction with p53. PML can indeed bind to p53, and affects its post-translational modifications such as acetylation, thus influencing its proapoptotic functions (Guo et al., 2000). Although PML does not possess any HAT activity, it actively recruits both CBP/p300 and HDAC1 to PODs (LaMorte et al., 1998; Wu et al., 2001), and therefore could exert an acetylating function indirectly.

Another post-translational modification of p53 that could be affected by PML is phosphorylation, since it has been recently shown that the homeodomain-interacting protein kinase 2 (HIPK2), a member of a novel family of nuclear serine/threonine kinases, binds to and activates p53 by directly phosphorylating it at Ser 46 (D'Orazi et al., 2002; Hofmann et al., 2002b). This kinase colocalizes together with p53 and PML in PODs, and modifies PML itself (Engelhardt et al., 2003). Finally, as previously mentioned, PML

seems to directly interact with the main regulator of p53 stability, the Mdm2 ubiquitin ligase (Wei et al., 2003). As for the p53-independent role of PML in the DNA damage response, it is not clear which pathway might be involved, but PML is itself a target of chk2 phosphorylation (Yang et al., 2002), suggesting that it might have a role as a downstream effector of the DNA repair cascade.

A protein that seems to play a critical role in p53 independent apoptosis mediated by PML is DAXX, which was originally cloned as a FAS-interacting protein, and found to act as a positive mediator of FAS- and TGFβ-induced apoptosis (Perlman et al., 2001). In $PML^{-/-}$ cells, the ability of DAXX to trigger apoptosis and to potentiate the FAS proapoptotic signal is markedly impaired (Zhong et al., 2000b). This is because, in PML+/+ cells, upon mitogenic activation, DAXX is dramatically upregulated, and accumulates in PODs where it physically interacts with PML and is sequestered by it (Li et al., 2000; Zhong et al., 2000b); as a consequence of this sequestration PML blocks the transcriptional repressive activity of DAXX that is necessary to trigger apoptosis. Interestingly this localization has been recently shown to be upregulated by ZIPK (Zipper-Iinteracting Protein Kinase), a proapototic kinase, which recruits DAXX to PODs via direct phosphorylation (Kawai et al., 2003). As expected, in APL cells the presence of PML-RARα results in DAXX dispersion and in enhanced DAXX transcriptional repression which consequently impairs its pro-apoptotic function (Li et al., 2000). Recently, however, contradictory results have been published on this matter, since Daxx silencing by short interfering RNA (siRNA) has been shown to sensitize cells to apoptosis (Chen and Chen, 2003), raising the question as to the real function of this nuclear factor.

The role of PML in proliferation and cellular senescence.

Once again the best evidence that PML is involved in the control of cell proliferation comes from $PML^{-/-}$ cells (Wang et al., 1998a), which divide more rapidly than their wild-type counterpart. Interestingly, $PML^{+/-}$ MEFs display an intermediate proliferation rate, suggesting that PML is haploinsufficient and needs homozygosity in the cells for growth suppression. The S phase population of $PML^{-/-}$ MEFs is increased with a concomitant decrease in the G_0/G_1 population and a similar increased proliferative rate is also observed in primary $PML^{-/-}$ splenocytes, thymocytes, and keratinocytes (Wang et al., 1998b). PML growth suppressive capacity is further confirmed by overexpression studies in tumor cell lines from various histological origins, where PML acts by inducing a block in the G1 phase of the cell cycle (He et al., 1997; Le et al., 1998).

Recent studies have pointed to PML as being a crucial player in regulating cellular senescence. PML was indeed shown to be upregulated by the activation of Ras, which is a known inducer of senescence (Serrano et al., 1997), leading to a dramatic increase in the size and number of PODs (Ferbeyre et al., 2000). PML overexpression, however, is itself sufficient to promote premature senescence in primary cells, and similarly to Ras, PML increases the levels of p16, hypophosphorylated pRb, S15 phosphorylation and K382 acetylation of p53, and the consequent upregulation of p53 target promoters (Ferbeyre et al., 2000; Pearson et al., 2000). Overall, this data demonstrate that PML can act downstream of RAS in this pathway. What is interesting, however, is that only a specific PML isoform (PML IV) seems to specifically induce premature senescence, and not surprisingly, the same isoform has been shown to interact with both p53 and pRb (Alcalay et al., 1998; Fogal et al., 2000). During RAS-induced cellular growth arrest, both pRb and p53 colocalize with PML in PODs (Pearson et al., 2000), although this does not seem to be

essential for PML mediated senescence (Bischof et al., 2002). Surprisingly, however, PML IV needs the cooperation of other PML isoforms, since the reintroduction of PML IV alone into a PML null background, does not induce senescence (Bischof et al., 2002). The mechanism involved in PML induced senescence thus still remains to be fully elucidated. Finally, the conclusive evidence that PML, like p53, is essential for the induction of cellular senescence upon oncogenic transformation comes from the observation that in $PML^{-/-}$ cells, oncogenic Ras induced senescence is drastically impaired (Pearson et al., 2000). Taken together, these findings support a model by which Ras upregulates PML levels, which in turn facilitates p53 activation, resulting in the upregulation of p21 and subsequent growth arrest. In support of the model, the ability of Ras to induce the p53 target gene *p21* is also impaired in $PML^{-/-}$ cells (Pearson et al., 2000).

Besides p53, another potential mediator of PML induced senescence is pRb, which also interacts with PML IV (Alcalay et al., 1998) and whose activity has been recently shown to be enhanced by PML (Khan et al., 2001b). PML has been found interacting with repressive complexes containing class I histone deacetylases, such as the N-CoR/SMRT-mSin3-Ski-HDAC1 complex, that is required for transcriptional repression mediated by pRb (Khan et al., 2001b). Overexpression of PML thus enhances such Rb-mediated repression, as well as other tumor-suppressor-mediated repressor complexes (Khan et al., 2001a; Khan et al., 2001b). Furthermore, PML-RARα can antagonize this function, and in $PML^{-/-}$ MEFs, the ability of both pRb and Mad to repress transcription is markedly impaired (Khan et al., 2001a; Khan et al., 2001b).

PML and the interferon response

Interferons (IFNs) are a large family of multifunctional secreted proteins that regulate

cellular anti-tumour and anti-viral responses. They can be classified into class I (α, β, ω and τ) and class II (γ) depending on their antigenic properties and their different ability to activate genes expression. In particular class I IFNs are acid stable, they are produced in response to viral infection by leukocytes (α) and fibroblasts (β) and activate genes by a membrane-nucleus cascade (Jak/STAT pathway) resulting in the binding of activated STATs (Signal Transducers and Activators of Transcription) to the IFN α/β Stimulated Response Element (ISRE) (GAAAN(N)GAAA) (Stark et al., 1998). Class II IFNs are, in contrast, acid labile. They are produced by activated T-lymphocytes and Natural killers cells (NKs) and they originate a cascade resulting in STATs binding to the IFNγ-activated site (GAS) (TTNCNNNAA) (Pfeffer et al., 1998). Although the two classes of IFNs have distinct celluar effects it is noteworthy to say that there is crosstalk between the two cascades (Der et al., 1998; Stark et al., 1998; Foss and Prydz, 1999).

The PML promoter has both ISRE and GAS sequences, and is therefore IFN inducible (Stadler et al., 1995). At the morphological level IFNs produce an increase in the size of PODs, increasing the amount of PML and of other resident proteins (Chelbi-Alix et al., 1995; Lavau et al., 1995). This observation led researchers to examine the importance of PML in the IFN response and once again, the PML knock out mice gave a very clear answer to this question: $PML^{-/-}$ cells are resistant to IFN induced apoptosis (Wang et al., 1998a). Further work was then orientated towards the antiviral effects mediated by IFNs. In particular a very clear study has recently demonstrated that the anti-HSV (Herpes Simplex Virus) state induced by exogenous IFN is mediated by PML. This effect is lacking in the $PML^{-/-}$ cells and the a viral protein, ICP0, disrupts PODs in order to preclude the establishment of a such antiviral state (Chee et al., 2003). The specific effects of ICP0 will be considered below.

As mentioned before, however, PML is not the only protein present in PODs that is regulated by IFNs and Sp100, Sp140, Sp110, ISG20 and PA28, besides PML, are all upregulated by IFNs (Regad and Chelbi-Alix, 2001); thus other proteins that become dispersed in $PML^{-/-}$ cells could also play an essential role in this pathway.

PML and viruses

As just noted, PML is upregulated in response to IFNs, and PODs play a pivotal role in the pathway. Thus it is not surprising that many viruses have evolved a protein that is able to interfere with PODs integrity or PML functions. Several reports have indeed shown that early during viral infection, different viral proteins transiently colocalize with PODs before altering their structure or composition (Everett and Maul, 1994; Ahn and Hayward, 1997; Sternsdorf et al., 1997a), and these will be described below.

The **Herpesvirus-1 (HSV-1) ICP0** protein was the first viral protein described to interact with PML Oncogenic Domains (Maul et al., 1993; Maul and Everett, 1994). Subsequently, other Herpes Viruses were also found to encode similar proteins, with related functions (Parkinson and Everett, 2000; Parkinson and Everett, 2001). ICP0, like PML, is a RING finger protein, and is involved in general transcriptional activation, promoting reactivation from latency and initiation of the lytic phase of the viral life cycle (Everett, 2000b). ICP0 appears to do so by stimulating the degradation of a number of cellular proteins via the ubiquitin-proteasome pathway (Everett, 2000a; Everett, 2000b). The interaction between PML and ICP0 results in the initial colocalization of the two proteins, followed by a reorganization of PODs structures. ICP0 is able to induce the proteasome degradatation of

the SUMOylated forms of PML, although the requirement for SUMOylation remains controversial: the higher specificity for the modified forms could simply depend on the spatial localization of the two proteins (Everett et al., 1998a; Chelbi-Alix and de The, 1999; Parkinson and Everett, 2000). Nonetheless, what has been recently demonstrated is that ICP0 is an E3 ubiquitin ligase (Boutell et al., 2002), that it uses UbcH5a as an E2 enzyme (Gu and Roizman, 2003), and that it requires PML Sumoylation at position 160 to exert an efficient degradation (Boutell et al., 2003). This activity is mediated through its RING finger domain and correlates with ICP0 function in the viral life cycle (Boutell et al., 2002). Blocking PML degradation by the addition of specific proteasome inhibitors results in both PML rescue and the failure of ICP0 to induce a lytic phase (Everett et al., 1998b).

One last feature that is worth mentioning is that HSV-1 genomes associate with the periphery of PODs, at very early stages after infection, and colocalize with ICP0 before the latter modifies these nuclear structures. The proteins and domains involved in this localization have been recently characterized, and seem to involve the immediate-early proteins ICP4 and ICP27, and their concomitant binding to DAXX and to the viral DNA (Tang et al., 2003). Viral replication compartments were shown to develop from these sites, and to be coupled to transcription (Sourvinos and Everett, 2002). Similar findings have also been reported for other viral systems, such as HPV, SV40, CMV and Adenovirus, suggesting that viral genomes have a tendency to be recruited to the periphery of these structures (Ishov and Maul, 1996; Maul et al., 1996; Ishov et al., 1997; Swindle et al., 1999). The reason for this recruitment is still largely unknown, but it may be an anti-viral control function exherted by PODs, which is supported by the aforementioned IFN upregulation. In agreement with this hypothesis ICP0-deficient viruses are particularly susceptible to inhibition by interferon (Mossman et al., 2000) and IFN treatment can inhibit

both viral immediate-early gene expression and ICP0-induced disruption of PODs (Taylor et al., 2000).

Cytomegalovirus (CMV), similarly to HSV-1, encodes proteins that disrupt PODs early after infection (Kelly et al., 1995), but unlike HSV-1 ICP0, the CMV **Immediate Early IE1** and **IE2** proteins are not ubiquitin ligases, and consequently have a different mechanism to fulfill their functions. Specifically, it has been demonstrated that both CMV IE1 and IE2 colocalise transiently with PML in punctate bodies at very early times after infection (2 h post-infection). By 4 h post-infection, the IE1 protein is distributed throughout the nucleus, and this correlates with a complete disruption of PODs (Korioth et al., 1996; Ahn and Hayward, 1997; Ahn et al., 1998). In contrast, the IE2 product retaines a punctuate pattern of expression even after PODs disruption (Ahn and Hayward, 1997). This was further confirmed in transient expression assays, where overexpression of IE1 alone produced the redistribution of PML into a uniform nuclear diffuse pattern, whereas overexpression of IE2 alone resulted in stable colocalization of the IE2 protein with PML in the PODs (Ahn and Hayward, 1997; Wilkinson et al., 1998). The mechanism by which IE1 disrupts PODs is not yet fully understood, however physical interaction and sequestration seems to account for most of it (Ahn et al., 1998).

To conclude with the herpesviruses, it should also be mentioned that the gamma sub-family members **EBV** (Epstein-Barr Virus)and **HHV-8** (Human Herpesvirus-8) also encode proteins able to colocalize with PML. In particular **EBV EBNA-5** is present in PODs where it colocalizes with CBP, a transcriptional co-activator (Szekely et al., 1996; Bandobashi et al., 2001). While EBNA-5 does not reorganize PODs structure, another protein, **BZLF1**, is able to do so by competing with PML for SUMO-1 (Adamson and Kenney, 2001). PODs disruption is, also in the case of EBV, linked to the lytic phases of

the viral life cycle (Bell et al., 2000), and we will see later how this also relates to HPV infection.

HHV-8 encodes an early protein, **K8 (K-bZIP)**, that is able to colocalize with PODs and to recruit p53 at these sites, possibly modulating its functions during the lytic phase of the viral life cycle (Katano et al., 2001). So far however no HHV-8 encoded proteins have been identified that disrupt PODs integrity.

Adenovirus infection causes a drastic redistribution of PML from spherical nuclear bodies into fibrous structures (Carvalho et al., 1995; Doucas et al., 1996). The product encoded by adenovirus **E4 ORF3** is responsible for this reorganization and is found to colocalize with PML into these fibers (Carvalho et al., 1995; Doucas et al., 1996). Interestingly, Adenoviral mutants carrying a deletion in E4 ORF3, are defective in viral replication as well as in the accumulation of late viral mRNAs, further connecting PODs disruption with the progression of the viral lytic cycle (Doucas et al., 1996). In addition, **E1A** accumulates in PODs, both during infection and in transient transfection assays. This association requires the pocket protein interaction domain, and although its biological significance still remains unclear, a role in preventing PML induced senescence, through reduced p53 phosporylation, might be one of the possible outcomes (Ferbeyre et al., 2000).

Similarly to E1A, the **SV-40 large T antigen**, is also found in close association with PODs, where it colocalises with p53 (Carvalho et al., 1995; Jiang et al., 1996). However it appears to require viral DNA for correct ND10 localization, and it is at these sites that T-antigen-directed replication apparently takes place (Tang et al., 2000).

Last, but not least, there is a growing number of reports linking **Papillomaviruses** to PODs structures. The first studies were with BPV, whose **L2** minor late protein was shown to

actively relocalise the major capsid protein **L1** to PODs (Day et al., 1998). L2 also had a similar effect on the distribution of the viral transcriptional activator E2, that shifted from a diffused nuclear distribution, to a punctuate one, in the presence of L2 (Day et al., 1998). These preliminary findings suggested PODs as possible sites of viral assembly, thus being targeted by the late viral proteins in order to increase the local concentration of virion components. Further studies were carried out on the HPV-33 L2 protein, showing that L2 is able to alter the structure of these nuclear bodies by dispersing PODs components such as Sp100 and recruiting others such as DAXX, without affecting PML localization (Florin et al., 2002b). Indeed DAXX recruitment seems to be the driving force for L2 accumulation into PODs (Becker et al., 2003). Interestingly these changes caused by L2 are necessary for L1 to be recruited to PODs, indicating that L2 is an essential mediator of viral assembly (Florin et al., 2002a).

These initial data pointed at the possibility that viral replication was coupled to viral assembly. This hypothesis was further strengthened by the results of studies done on HPV-11 by Swindle and coworkers. In their system **E1** and **E2** colocalized with cellular Replication Protein A (RP-A) as well as with PML in a proportion of cells, and these sites of colocalization were found positive for bromodeoxyuridine (BrdU) incorporation (Swindle et al., 1999). Unexpectedly, RP-A, E1, and E2 also colocalised in nuclear foci in the absence of an HPV origin-containing plasmid, suggesting a reorganization of the cellular transcription machinery by these two proteins that is independent of viral DNA (Swindle et al., 1999). It is important at this stage to mention that the HPV genome is not always linked to PODs. The E2 protein has been shown to link HPV genomes to cellular mitotic chromosomes to ensure proper segregation to daughter nuclei (Bastien and McBride, 2000) and possibly E2 may also well play a similar role during the productive

stages of viral infection, linking the genome to PODs structures, where replication takes place.

One recent report has linked the **E4** protein of HPV1 to PML. These two proteins were found to colocalise in HPV1-induced warts, and as a consequence of this interaction PML was relocalized to E4 inclusions. These findings demonstrate that nuclear PML-E4 inclusions are indeed characteristic of late stage infection, suggesting that reorganization of PML might be an essential step for the HPV life cycle as was previously reported for the other DNA viruses (Roberts et al., 2003).

Taken together, we can conclude that PODs represent a preferential target for DNA tumor viruses, therefore strengthening the involvement of these structures in oncogenic processes and in antiviral defense.

Getting in and out of the nucleus

So far I have described the complex organization that is present within the nucleus. As noted at the beginning however, the first level of complexity in the cell is maintained by membranes, and consequently by the regulation of the transit between one compartment and the other. The nucleus is surrounded by a **nuclear envelope**, a double-membrane structure, whose **outer membrane** is closely associated with the rough endoplasmic reticulum (RER) and, similarly to the RER, it contains ribosomes on its surface. The **inner membrane** is in close contact with the **peripheral nuclear lamina,** which is a structure

composed of lamins A/C and B, which is thought to play a role in regulating nuclear envelope structure and anchoring interphase chromatin at the nuclear periphery (Holaska et al., 2002). The nuclear lamina is indeed disrupted at each mitosis by an APC (Anaphase Promoting Complex) dependent mechanism, and then reforms early in G1 (Hixon and Gualberto, 2000).

The connection between the nucleus and the cytoplasm is ensured by the Nuclear Pore Complexes (NPCs), one of the biggest macromolecular structures in eukaryotic cells. The NPC penetrates the two lipid bilayers of the nuclear envelope and can accommodate a large number of diverse RNA and protein cargoes, ranging in mass from a few kDa to almost 50 MDa (Rout and Aitchison, 2001; Vasu and Forbes, 2001; Pante and Kann, 2002). Small molecules and ions can diffuse passively through the NPC, but macromolecules larger than about 50 kDa require a facilitated mechanism (Rout and Aitchison, 2001; Vasu and Forbes, 2001). The pecularity of this system is that proteins can pass through the membrane in both directions, and thanks to a central acqueous environment they do not need to be unfolded. As expected from the large variety of transport substrates, a large class of nuclear transporters are present within a cell, and these fall into three families:

1) the family of **importin β-like** transport factors (importins/exportins or **karyopherins**)

2) the class represented by the small nuclear transport factor 2 **NTF2/p10**, which imports the small GTPase Ran into the nucleus (Ribbeck et al., 1998; Smith et al., 1998)

3) the **Mex67/Mtr2** heterodimer which represents the third class of transporters and is involved in the nuclear export of mRNA (Conti and Izaurralde, 2001).

1) **Karyopherins** comprise a large number of mobile transporter proteins with molecular

masses ranging from 90 and 130 kDa. Their main characteristic is the presence of several HEAT repeats. These acidic alpha helical motifs are about 50 aminoacids long and are present in tandem, to give multiple protein-protein docking sites. Structurally the Karyopherins' HEAT repeats form two domains, an amino-terminal one that binds the small GTPase Ran, in its GTP bound form (Chook and Blobel, 1999), and a carboxy-terminal one with a cargo-binding domain (Cingolani et al., 1999; Vetter et al., 1999; Cingolani et al., 2002). The difference between import and export transporters is defined by the ability of the HEAT domain to bind cargoes cooperatively with Ran-GTP, in the case of exportins, while importins release their cargoes when bound to Ran-GTP. Ran is thus the key component that defines compartment identities for transport, and it's the Ran-GTP/Ran GDP gradient across the NPC that determines the vectorality of nuclear transport allowing the accumulation of proteins against their concentration gradients (Kuersten et al., 2001). Thus Ran is in its GTP bound form when in the nucleus, while the Ran-GDP pool is higher in the cytoplasm (Yoneda et al., 1999). Cargo proteins bound by karyopherins have a conserved stretch of aminoacids that defines their nuclear localization signal (NLS). The "classical NLS" was the first to be identified, and is characterized by a string of highly basic amino acids, like Arginines or Lysines (Dingwall et al., 1982). Classical NLS can also exist as tandem repeats, with a variable spacing between them: in this case they are termed Bipartite NLS, and bind importins with a different specificity as compared to classical NLS (Strom and Weis, 2001). Recently, new NLS sequences have been identified that do not conform to the classical or bipartite NLS consensus motif. An example is the M9 sequence of the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein (Siomi and Dreyfuss, 1995), which is recognized by transportin (karyopherin-2)(Nakielny et al., 1996) and is a glycine rich motif, with no similarity to the other previously reported NLS (Siomi and Dreyfuss, 1995).

2) The **NTF2/p10** protein, as mentioned before, shuttles Ran in and out of the nucleus, allowing maintenance of a gradient between the GTP and the GDP bound form, that is essential for importin-mediated nucleo-cytoplasmic shuttling (Ribbeck et al., 1998; Smith et al., 1998). The nuclear localization of the Ran guanine-nucleotide exchange factor, **RanGEF**, is also believed to help in maintaining nuclear Ran in a GTP-bound form. Ran GTPase-activating protein (**RanGAP**) is instead localised on the cytoplasmic side of the nuclear pore, ensuring that cytoplasmic Ran is in its GDP bound form.

3) The third transport receptor family is involved in the nuclear export of mRNA. This mRNA exporter is a heterodimer of a large, conserved subunit named **Mex67** and a small subunit termed **Mtr2** (Conti and Izaurralde, 2001) (Reed and Hurt, 2002). There is no evidence for a direct involvement of Ran in Mex67 mediated shuttling, and the mechanism providing directionality to mRNA export, still remains largely unknown.

Interestingly, these three classes of nuclear receptor do not share any significant degree of homology in terms of structure, but they have all evolved to shuttle in and out of the nucleus, and to interact with the NPC.

Human Papillomavirus

So far I have described the complex organisation of eukaryotic cell nucleus. For the remainder of this introduction, I will describe how HPV infection, and in particular how the three viral oncoproteins E5, E6 and E7, affect this organisation.

HPV general features

Human Papillomaviruses (HPVs) are double stranded, exclusively epitheliotropic, differentiation dependent viruses which are linked to the development of a number of human malignancies (zur Hausen, 1991). These include squamous cell carcinomas (SCCs) in immunosuppressed individuals and in patients with epidermodysplasia verruciformis (EV), head and neck cancers, anogenital cancers and, most notably, carcinoma of the uterine cervix (de Villiers, 1997). HPVs can be divided into two groups depending on whether they infect mucosal epithelia (ano-genital or laryngeal) or cutaneous epithelia. Moreover, of the 130 HPV types so far identified, only a small subset of virus types is associated with the aforementioned malignancies. These types are termed "high-risk" as opposed to those types associated with benign condylomas of the uterine cervix and laryngeal papillomas, or plantar and palmar warts, and are hence termed "low-risk". (zur Hausen, 1999).(summarized in Table 2). The difference between these two groups of HPVs, high and low risk, and the role played by high-risk in the initiation and progression of malignant diseases has been the subject of intense study for more than a quarter of a century. Two aspects of HPV research appear to have a bearing on this: first the spectrum of cellular proteins to which the viral E6 and E7 oncogenes bind, and second, the regulation of the cellular localization of these viral proteins. This latter consideration is the subject of this thesis and will be discussed below.

Table 2.

HPV 1	Cutaneous	Low-risk	Plantar skin warts
HPV 2, 27	Cutaneous	Low-risk	Common skin warts
HPV 3,10	Cutaneous	Low-risk	Flat skin warts
HPV 5, 8, 17, 20	Cutaneous	High-risk	Benign and malignant epidermodysplasia verruciformis lesions
HPV 1-6, 8, 10, 11, 14-16, 18-20, 23-25, 27, 29, 36, 38, 41, 47, 48	Cutaneous	Low-Risk High-risk	Skin cancer in renal transplant patients
HPV 6, 11	Mucosal	Low-risk	benign anogenital lesions, oral papillomas, laryngeal papillomas, conjunctival papillomas
HPV 13, 32	Mucosal	Low-risk	Focal epithelia hyperplasia
HPV 16, 18, 31, 33, 45	Mucosal	High-risk	genital intraepithelial neoplasia and cancers

The epithelial tropism of HPV and its dependency upon host-cell differentiation for viral replication, have two important consequences: the first concerns the viral life cycle, requiring on the one hand keratinocyte differentiation, but on the other hand keeping the host cell in the cell cycle, thereby leading to an aberrant epithelial state; I will describe at a later point how this is achieved.

The second is an exclusively experimental problem, since there is not a generally usable in vitro system that allows culture of the virus, this has driven HPV research to become mainly molecular. The molecular approach to studying the interplay between viral and host proteins has provided a wealth of information on normal cell regulatory pathways and functions. Although studying those interactions that lead to host cell malignancy are of critical importance for a better understanding of the process of malignant disease, and how it may be treated under clinical conditions, studying those interactions that are not directly linked to tumorigenicity continues to provide us with crucial data on viral regulation of transcription, replication and cellular trafficking.

This thesis describes how intracellular localization is an essential regulatory element for certain viral proteins, which can target many key cellular regulatory proteins through gaining access to specific nuclear structures (described in the first section of my introduction). From this data we also gain a better understaning of the cellular regulatory pathways that are controlled by these structures.

Genome organization and the infectious viral life cycle

HPVs are extremely appealing from the molecular point of view, because they are small double stranded DNA viruses, with a circular genome of approximately 7.9 Kb, encoding only 6 non structural, early proteins (E1, E2, E4, E5, E6 and E7) and two structural proteins (L1 and L2) that form the capsid. The degree of conservation of genomic organisation among the different genotypes is high, and together with the two early and late regions that are temporally and spatially differentially regulated, there is a non coding upstream regulatory region (URR) of approximately 1 Kb, where viral and cellular factors bind to modulate transcription and replication (**Fig. 3B**).

Many studies have focused on the peculiar dependency of the HPV life cycle on cellular differentiation. This requirement is probably linked to the spatial distribution within the stratified epithelium of specific transcription factors such as the tissue-restricted POU domain transcription factors, which bind octamer or octamer-like gene sequences or the Yin-Yang1 (YY1) protein. Initial studies demonstrated a negative regulation by Oct-1 and YY1 on HPV gene expression (Hoppe-Seyler et al., 1991; Bauknecht et al., 1992; Ai et al., 2000), and more recent reports point at a clear regulation of HPV promoters by these factors. Skn-1a is an octamer binding protein and is primarily expressed in suprabasal cells of the epidermis, it binds the HPV URR, driving activation in a sequence specific manner

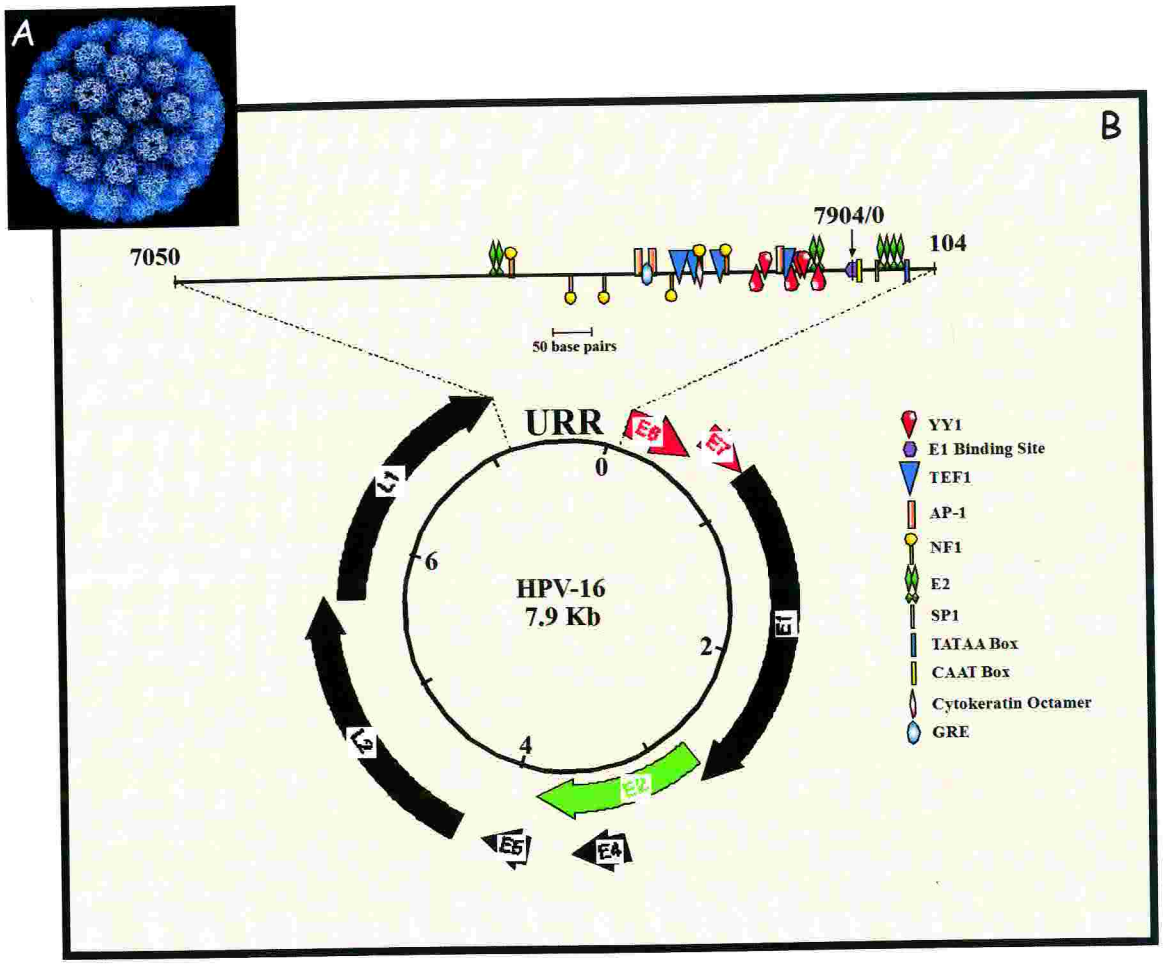


Fig. 3 PanelA. HPV Capsid structure.
Panel B. Genome organisation of Human Papillomavirus type 16. Viral Open Reading Frames (ORFs) are shown. E: Early proteins, L: Late proteins. The Upstream Regulatory Region (URR) is shown with the different transcription factors binding sites. HPV Early Proteins are transcribed from a major early promoter (P97) within the URR.

(Andersen et al., 1997). YY1 is a sequence-specific DNA-binding transcription factor that has many important biological roles activating or repressing many genes during cell growth and differentiation (Petkova et al., 2001; Kurisaki et al., 2003). Previous studies have established that YY1 interacts with histone acetyltransferases p300 and CREB-binding protein (CBP) as well as with histone deacetylase 1 (HDAC1), HDAC2, and HDAC3, being itself (YY1) regulated through acetylation/deacetylation by these cofactors (Yao et al., 2001). Moreover deletion of the murine YY1 gene results in embryonic lethality, suggesting an essential function for YY1 in development (Donohoe et al., 1999). Recent data strongly suggest that YY1 and Skn-1a play an antagonistic role: the transcription from the HPV-16 P670 late promoter is indeed repressed primarily by YY1 binding to two sites, and the displacement of YY1 by hSkn-1a releases the promoter from such repression (Kukimoto and Kanda, 2001). This may thus provide a molecular link between HPV gene expression and epidermal differentiation. Other transcription factors such as AP-1, NF1, SP1, TEF-2, NFA and the glucocorticoid receptor also bind to the URR (Gloss et al., 1987; Gloss and Bernard, 1990; Thierry et al., 1992; Butz and Hoppe-Seyler, 1993). It has been suggested that the epithelial specificity of the HPV enhancer is brought about via binding sites for these supposed ubiquitous transcription factors that synergistically cooperate. The specificity resides in the fact that they vary in concentration between different cell types and cell-specific interactions, residing outside the DNA-binding domain, can occur (Chong et al., 1991).

Although the exact process by which both cellular differentiation and transcription factors regulate the productive life cycle still remains to be investigated, what is thought to be the key event, is the switch from an early promoter to a late promoter, although the mechanism by which this is regulated by the virus still remains unclear. Especially since E2 has been shown to have no repressive effect on an episomal viral copy, while it strongly repressed

E6 and E7 expression when integration had occurred. Thus it seems likely that any effect of the E2 protein on the expression of the E6 and E7 genes during the normal viral life cycle is of secondary importance compared to the function of E2 in replication (Bechtold et al., 2003).

The transcription of E1 and E2 is driven by the early promoter, and this promotes viral DNA replication (Stubenrauch and Laimins, 1999). In order to function, **E1** and **E2** form heteromeric complexes and individually bind specific sequences within the viral origin of replication. E1 alone binds an A/T-rich sequence with low affinity, while E2 binds with high affinitiy to specific E2 binding sites within the URR (ACC(N)6GGT) some of which are adjacent to the origin of replication (Bedrosian and Bastia, 1990). E2 is also able to bind E1 which has an intrinsic helicase and ATPase activity (Hughes and Romanos, 1993) and this allows the formation of a multimeric complex with low sequence specificity, loaded onto a specific sequence in the DNA (Sedman and Stenlund, 1995; Sanders and Stenlund, 1998). All the other replication proteins, including DNA polymerase α-primase, are derived from the host cell and E1 is responsible for their specific recruitment (Park et al., 1994; Masterson et al., 1998).

Where does the infection take place?

Initial infection probably takes place in basal layer stem cells. The receptor mediating entry of the HPV capsid (**Fig. 3A**) has not to date been clearly identified, however initial in vitro results suggested that papillomavirus capsids could bind a widely expressed and evolutionarily conserved cell surface receptor (Roden et al., 1994). Later studies pointed at α6 integrin and in particular at complexes containing α6 integrin complexed with either β1 or β4 integrins as putative receptors for PV binding and entry into epithelial cells (Evander

et al., 1997). This integrin is an attractive candidate since it's expressed on epithelial stem cells (Li et al., 1998) and is particularly present during wound-healing, a state that would allow the virus to bypass the epithelial physical barriers. Moreover, HPV interacts with heparin and with cell-surface glycosaminoglycans (GAGs) (Joyce et al., 1999), and this could provide an initial low affinity binding followed by a higher affinity integrin receptor which would, in turn, mediate internalisation. Notably, both studies were conducted using Virion Like Particles (VLPs) containing only **L1**, the major capsid protein, excluding **L2**, the minor capsid protein, from a possible role in receptor binding, and thus this may not reflect the *in vivo* situation.

Within the epidermis, cell proliferation takes place in the basal layer of keratinocytes that are attached to an underlying basement membrane. Cells that leave the basal layer undergo terminal differentiation as they move towards the tissue surface. The basal layer contains two types of proliferative keratinocyte: stem cells, which have unlimited self-renewal capacity, and transiently amplifying cells that are destined to withdraw from the cell cycle and terminally differentiate after a few rounds of division (Watt, 2001). After capsid internalisation has occurred into either true epithelial stem cell or transiently amplifying cells, HPV genomes are maintained as episomes at a stable number that ranges from 50 and 100 copies. The DNA remains in the nucleus of the infected cell and is equally distributed to daughter cells (Stubenrauch and Laimins, 1999). Transiently amplifying cells go on to replicate for several cycles, before producing daughter cells which will commit to differentiation (Watt, 1998). In the basal layer HPV DNA can be maintained stably for several years and it is now clear that HPV infections are extraordinarily common and are normally held in a sub-clinical state by functional immune systems thus entering into a so called "latent-phase". However HPVs can be reactivated by immunosuppressive conditions (Ferenczy et al., 1985; Maran et al., 1995; Broker et al., 2001). Transient replication of

HPV genomes requires only E1 (Gopalakrishnan and Khan, 1994), but E1-dependent replication is stimulated by the E2 protein. However stable maintenance in normal keratinocytes requires also E6 and E7 expression suggesting a role for E6 and E7 in maintaining the episomal state of the HPV genome (Del Vecchio et al., 1992; Thomas et al., 1999).

In normal epithelia, as cells divide and move up from the basal layer, daughter cells exit the cell cycle and commit to differentiation. However the dependency of HPV on differentiation specific cellular factors for replication, has an important consequence: the keratinocytes commitment to differentiate becomes uncoupled from their exit from the cell cycle. This produces two general categories of epithelial change (see **Fig. 4**): the first is a viral cytopathic effect that occurs in the maturing, differentiating cells, which includes koilocytotic atypia. This results from execution of the normal viral life cycle, with suprabasal, differentiated cells, undergoing a last S-phase before packaging the viral genomes into capsids (Taichman et al., 1983). The second is an abnormality in the growth characteristics and differentiation of the epithelium that has its origins in a fundamental change in the replicating (basal/parabasal) cells. This phenomenon produces morphologic disturbances in all cell layers and is best documented in cancer-associated papillomaviruses (Crum et al., 1985), due to the fact that the replicative phase of high-risk viruses is confined to the differentiated layers of the epithelium, in cells that have completely exited the cell cycle and would normally be no longer permissive to replication (Doorbar et al., 1996).

The two viral proteins that are mainly involved in this deregulation are E6 and E7, and a consensus is emerging whereby E7 mainly stimulates G1/S transition and DNA synthesis while E6 blocks the cell from undergoing growth arrest and apoptosis due to this unscheduled re-entry into the cell cycle. A consequence of these specific roles is that during tumour formation E7 plays a critical role in tumour promotion, while E6 contributes

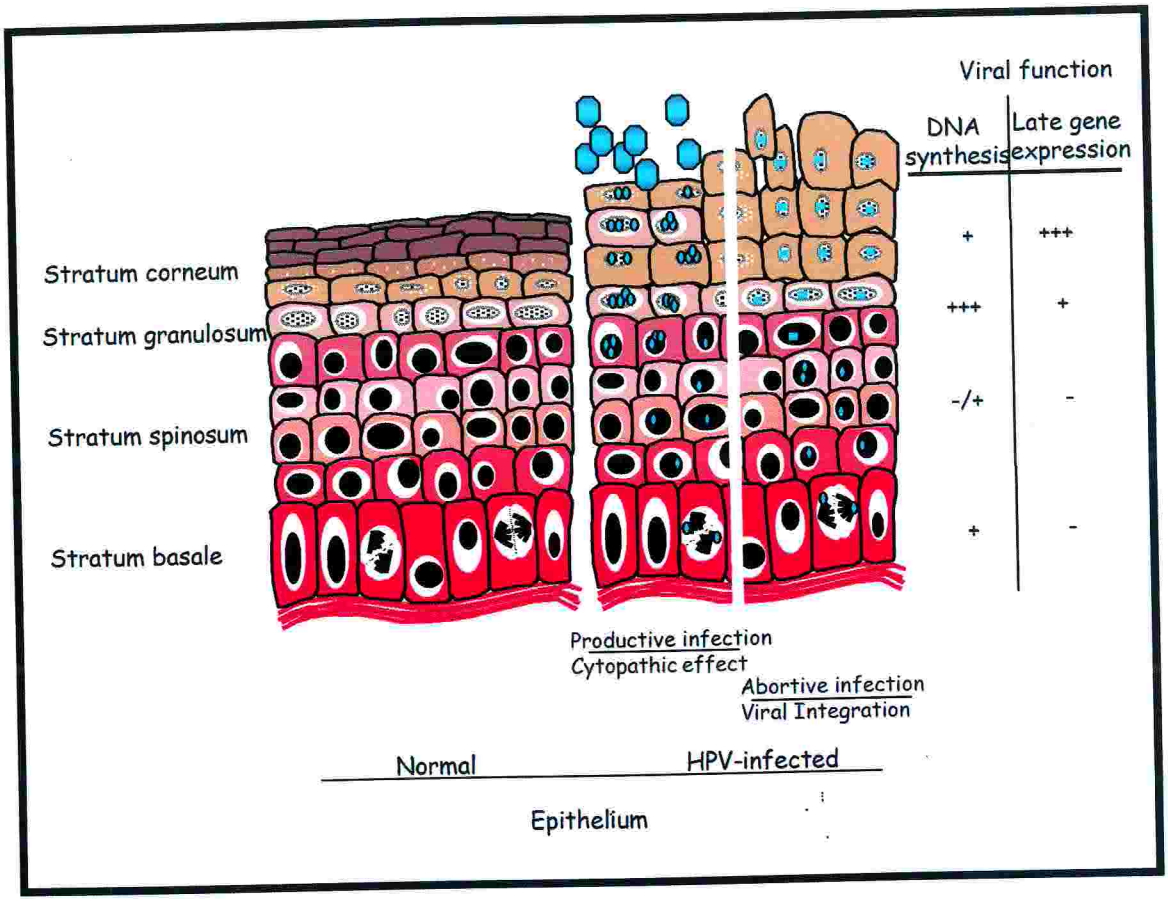


Fig. 4 Differentation dependent functions in HPV-infected epithelial cells. Typical HPV productive and abortive infections are shown in the right panel.

more during the later stages of tumour progression (Song et al., 2000; Riley et al., 2003).

HPV is a tumor virus.

As just noted, one of the possible outcomes of HPV infection is the degeneration into malignancy. The most studied of the HPV associated tumours is probably cervical cancer, which is the 4th most common cancer in developed countries, and the second in developing countries (Pisani et al., 2002). The association between this type of cancer and HPV has been established after 25 years of epidemiological studies showing that the viral DNA is present in around 99% of tissue biopsies (zur Hausen, 2002).

Molecular studies have narrowed down the oncogenic potential of the virus to specific viral ORFs, pointing to E6 and E7 as being the two major oncoproteins. In particular the continued expression of E6 and E7 in cervical tumours and derived cell lines is often detected many years after the initial immortalizing events (Schwarz et al., 1985; Smotkin and Wettstein, 1986; Androphy et al., 1987; Banks et al., 1987) and their continued expression is required to maintain the transformed phenotype of rodent cells. Moreover, knocking out the expression of these two proteins by a variety of different techniques (Antisense/Ribozyme/blocking peptides/si-RNA) results in cell growth arrest and/or apoptosis (von Knebel Doeberitz et al., 1992; Alvarez-Salas et al., 1998; Butz et al., 2000; Jiang and Milner, 2002) .

Finally several studies have demonstrated that the E7 ORF is able to transform NIH 3T3 or Rat-1 cells (Bedell et al., 1987; Vousden et al., 1988), can cooperate with an activated ras oncogene to transform primary baby rat kidney cells (Matlashewski et al., 1987; Phelps et al., 1988), and together with E6 is also able to extend the life span of human foreskin keratinocytes (Hawley-Nelson et al., 1989) (Hudson et al., 1990) (Munger et al., 1989a).

The Function of the HPV oncogenes

HPV E5

The HPV E5 proteins are small hydrophobic molecules approximately 10 kDa in size. They are type II transmembrane proteins, able to form homodimers (Bubb et al., 1988; Alonso and Reed, 2002), and cell fractionation studies have localised these polypeptides predominantly to the Golgi apparatus, the endoplasmic reticulum, and the nuclear and plasma membranes of transformed cells (Conrad et al., 1993). They lack any specific enzymatic activity, but nonetheless they act as functional modulators of cellular membrane proteins that regulate cell growth (DiMaio and Mattoon, 2001). Most of the early studies on E5 were done on the Bovine Papillomavirus encoded protein (BPV E5). This was because it has a higher transforming potential and less cell line dependency. Early studies showed that BPV E5 efficiently caused morphologic and tumorigenic transformation of murine fibroblasts, while HPV16 E5 was inactive in these assays. In contrast, HPV16 E5 was able to transform murine keratinocytes, suggesting an epithelial cell dependency for HPV E5 induced transformation (Schlegel et al., 1986; Leptak et al., 1991).

The major mediators of E5 activity are tyrosine kinases, and the involvement of this signalling pathway in transformation by E5 was indicated by several findings, with BPV E5 mainly affecting the PDGF pathway (Petti et al., 1991; Petti and DiMaio, 1992; Goldstein et al., 1994; Petti et al., 1997; Lai et al., 1998), while HPV E5's effect is maybe mainly mediated by the EGF cascade (Pim et al., 1992; Straight et al., 1993; Crusius et al., 1997).

Other possible functions of E5 are being investigated and results point at E5 as having an important role in the inhibition of apoptosis. In particular E5 seems to be involved in the

inhibition of ligand-mediated apoptosis, either by Fas ligand (FasL) or by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Kabsch and Alonso, 2002) and E5-expressing cells are protected from apoptosis through an enhancement of the PI3K-Akt and ERK1/2 MAPK signalling pathways (Zhang et al., 2002).

Finally, recent data suggest that HPV16 E5 may be able to decrease immune recognition of infected keratinocytes via down regulation of MHC class II protein expression, and it does so by preventing the breakdown of the Ii monomorphic peptide, thus blocking the formation of peptide-loaded, mature MHC class II dimers, exposed on the cell surface (Zhang et al., 2003).

Overall a consensus is emerging where E5 acts mainly during the early steps of immortalisation. This makes sense since the E5 ORF is often lost following the integration of the viral genome into the host cell chromosomes that frequently anticipates the development of cervical tumours, thus indicating that E5 has little or no role in the later stages of malignancy. However it is worth noting that, although E5 is frequently deleted, the EGFR genes are often amplified in cervical tumours, possibly compensating for E5 loss (Bauknecht et al., 1989; Kohler et al., 1989). As for the role of E5 in the viral life cycle, two recent papers have looked specifically at E5 inactivation in the context of the entire genome during differentiation. They demonstrated that HPV31 E5 modifies the differentiation-induced cell cycle exit and supports the ability of HPV31-positive keratinocytes to retain proliferative competence (Fehrmann et al., 2003). Similarly an HPV16-E5 mutant genome, was also described to have defects during the productive stage of the viral life cycle (Genther et al., 2003).

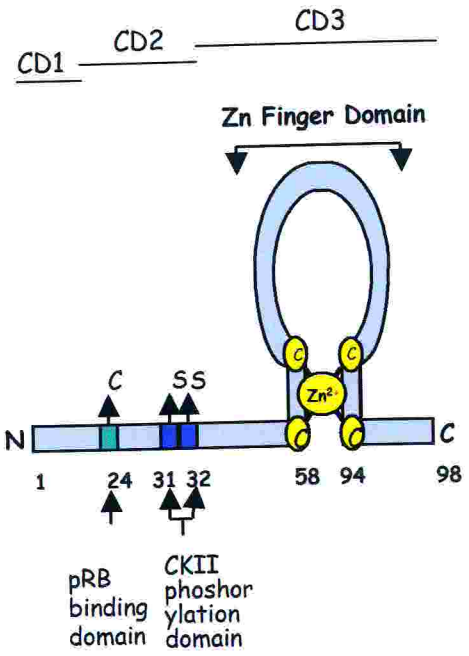
HPV E7

E7 is required for the of productive stage of the HPV life cycle. It functions by creating a favourable environment for HPV DNA synthesis, perturbing the keratinocyte differentiation program and inducing the host DNA replication machinery (Flores et al., 2000). High-risk, but not low-risk HPVs transcribe both E6 and E7 as bicistronic and polycistronic mRNAs (Schwarz et al., 1985; Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Chow et al., 1987) and the E7 ORF is, in particular, mainly transcribed from the E6*I/E7 transcript, the most abundant transcript in the lower strata of the infected epithelium (Shirasawa et al., 1991).

E7 Structure.

The E7 protein has a secondary structure very similar to that of the other oncoprotein, E6, the latter having two zinc-fingers, while E7 has only one, and both having similar spacing constraints (**Fig. 5**). This is intriguing from an evolutionary point of view, and has led researchers to hypothesize a possible evolution of the two ORFs by duplication (Cole and Danos, 1987). The E7 proteins are about 100 amino acids long and they have a N-terminal portion that shares homology with the conserved regions 1 and 2 (CR1 and CR2) of the Adenoviral E1A protein, and with regions in the Large Tumor Antigen (T Ag) of polyomaviruses (Phelps et al., 1988; Vousden and Jat, 1989). These regions of homology suggest similar functions, and indeed this region contains the Retinoblastoma Binding signature, the LXCXE motif (where X denotes a nonconserved residue) that allows them to bind and inactivate pRb functions (Chellappan et al., 1992). Hydrophobic and electrostatic

HPV E7 PROTEIN



E7 interacting cellular protein	Low Risk	High risk
pRb P107 p130	+	++
Cyclins/cdks inhibitors	+	++
TBP	+	++
skip	+	++
Mi2beta HDACs	+	+
M2-PK	+	+

Fig. 5 The HPV E7 protein. Schematic representation of E7 showing the N-terminal CD1, the CD2, encompassing thepocket proteins binding motif and the CKII Phosphorylation syte, and the CD3 region, whit the single zinc-finger.
On the right panel are summarized some of E7 binding partners.

properties are strongly conserved in this segment even though the non conserved amino acids vary considerably from one Rb-binding protein to another (Figge et al., 1993). On the basis of this homology with E1A, the E7 protein can be divided into three conserved domains (CD1, CD2, CD3).

The N-terminal CD1 (aas 1 to 20) shows high homology to the sequences of conserved domains 1 of adenovirus E1A and point mutations in this area lead to a reduced activity in cellular transformation (Banks et al., 1990; Watanabe et al., 1990; Phelps et al., 1992). This region does not directly bind Rb, although recent reports point at its first 20 aminoacids as being essential for the interaction with a large microtubule associated protein (MTAF600) that interacts with Rb and with many other cellular proteins (Howley-Munger-Nakatani personal communication). The CD2 (aas 21-40) contains the Rb binding motif (Munger et al., 1989b) and a Casein Kinase II (CKII) consensus site. E7 is indeed a phosphoprotein (Smotkin and Wettstein, 1987) and is mainly phosphorylated on the two Serines S31 and S32 by CKII (Firzlaff et al., 1989; Barbosa et al., 1990). This modification seems to be important for protein-protein interaction, since point mutants that abolish these modifications impair E7 binding to cellular proteins such as TBP, p300 and Skip (Massimi et al., 1996; Prathapam et al., 2001; Bernat et al., 2003). Other than CKII, E7 is also phosphorylated on S71 by another as yet unidentified kinase and this modification is also cell cycle regulated (Massimi and Banks, 2000). A similar CKII consensus is also found in polyomavirus large and middle T antigen and Ad E1A which are both phosphorylated by CKII (Schaffhausen and Benjamin, 1979; Vousden and Jat, 1989). In the case of E1A, a mutant in this region is defective in fully derepressing E2F targets in their natural chromatin context (Alevizopoulos et al., 2000).

The C-terminal portion, CD3 (aas 41-98) contains a classical Zinc Finger with two copies

of a Cys-X-X-Cys Zinc-binding motifs separated by 29 amino acids (Barbosa et al., 1989), and is responsible for dimerization/multimerization (McIntyre et al., 1993; Clements et al., 2000) as well as for the interaction with many cellular proteins such as the Mi2beta/HDAC1 complex (Brehm et al., 1999), the cyclin E and A/CDK complexes (Arroyo et al., 1993; McIntyre et al., 1996), TBP (Massimi et al., 1997), Skip transcriptional coactivator (Prathapam et al., 2001) and the M2-Pyruvate Kinase (Zwerschke et al., 1999) (summarized in **Fig. 5**).

E7 cellular localization

The HPV E7 protein has been found by immunofluorescence studies in a variety of cellular compartments depending on the different antibodies used in the study or to the particular cellular system. It is mainly a nuclear protein (Sato et al., 1989; Greenfield et al., 1991; Smith-McCune et al., 1999) although it has been detected in the cytoplasm (Smotkin and Wettstein, 1987; Fujikawa et al., 1994) and in the nucleoli (Zatsepina et al., 1997). A putative region responsible for nuclear entry has been identified between aminoacids 16 and 41, and nuclear entry seems to be independent of pRb binding, although no clear NLS is present in this region (Fujikawa et al., 1994). What is important to remember is that E7 targets are both in the nucleus and in the cytoplasm, thus it is possible that both reported localizations are true and may depend on cell cycle and, possibly, on differentiation (Zwerschke and Jansen-Durr, 2000).

Further explanations for the differences between reports on the pattern of expression of E7, are the lack of good antibodies and the relative low-abundance of the protein. As I will

describe in the results section, I made use of tagged proteins in order to look at E7 from different HPV types, thus avoiding problems due to the lack of type specific antibodies.

HPV E7 and its role in deregulating cell cycle control

The main role of E7 is to overcome cell cycle exit in cells that are nonetheless differentiating. In order to do this, E7 specifically targets key cellular proteins that control the cell cycle, inactivating their normal regulation by different means. The best known target of E7 is the Rb^{p105} tumour suppressor, and the other pocket proteins p107 and p130 (Munger et al., 1989b).

pRb is a large protein that was first identified as mutated in Retinoblastoma (Godbout et al., 1983) and is functionally inactivated in many human tumours either at the gene or protein level (Gorgoulis et al., 1998; Jarrard et al., 2002). Its main role is to control a specific Restriction point (R) that allows G1/S transition and leads the cell into correct S phase entry with controlled DNA synthesis and eventually cell division. These effects are mainly mediated by its interaction with the E2F transcription factor family, through which pRb mediates repression of key regulatory genes, although in recent years a more complex pattern of interactions has been emerging (Stevaux and Dyson, 2002). Briefly, there is a change in protein complexes, between repressor and activator E2F proteins, as well as between histone deacetylase and acetylase complexes that tightly regulate, through the pocket protein interactions, the progression through G1/S as well as later stages of the cell cycle (Takahashi et al., 2000; Frolov et al., 2001; Rayman et al., 2002).

In general terms, E7 perturbs this pathway by both binding to pRb and allowing E2F to

activate its target genes, as well as by promoting pRb degradation along the proteasome pathway (Munger et al., 2001). These two functions are certainly related, but only the latter correlates with the ability of E7 to induce cellular transformation (Helt and Galloway, 2001). In particular this study and others (Banks et al., 1990; Jewers et al., 1992; Phelps et al., 1992; Brokaw et al., 1994) have identified mutants of E7 that do not destabilise pRB, but nonetheless activate E2F promoters, similarly to the situation described for the non oncogenic HPV-1 and HPV-6 E7 proteins (Ciccolini et al., 1994; Schmitt et al., 1994; Armstrong and Roman, 1997). The regions identified by some of these mutants encompass the amino terminal CR 1 region of E7, and can now be possibly explained by recent findings showing MTAF600 binding to this region (Munger-Howley-Nakatani personal communication). Other mutants fall within the CKII consensus phosphorylation site immediately adjacent to the pRB binding site and a possible explanation could reside in the lack of ability of these mutants to activate E2F promoter in their natural chromatin context, as has been suggested for E1A mutants in the homologous region (Alevizopoulos et al., 2000).

As mentioned above, binding to the pocket proteins by E7 results in their degradation through the proteasome pathway (Boyer et al., 1996), and E7 itself is a target of ubiquitin-dependent proteolysis (Reinstein et al., 2000). The exact mechanism of pRB destabilization, however, remains unclear, and although E7 interacts with the S4 subunit of the 26S proteasome (Berezutskaya and Bagchi, 1997), this does not seem the correct link, since a mutant lacking this interaction domain, can still efficiently degrade the pocket proteins (Gonzalez et al., 2001).

HPV E7 interferes with the IFN response

One last feature of E7 that is relevant for the discussion of this thesis is its ability to interfere with the interferon pathway. HPV E7 is indeed able to abrogate the formation of the interferon-stimulated gene factor 3 (ISGF3) transcription complex, and it does so by binding to the p48 protein, the DNA-binding component of ISGF3, preventing its translocation into the nucleus following IFNα stimulation (Barnard and McMillan, 1999). Moreover E7 can also interact with IRF-1, a protein involved in the crosstalk between IFN γ and IFNβ signaling, thus inactivating both pathways (Park et al., 2000; Perea et al., 2000).

HPVE6

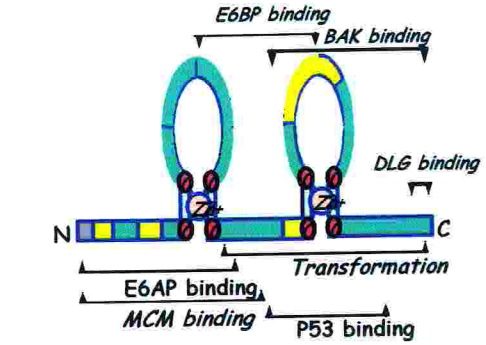
High-risk, but not low-risk HPVs transcribe the E6 ORFs as bicistronic and polycistronic mRNAs (Schwarz et al., 1985; Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Chow et al., 1987). The resulting transcripts encode the full length proteins and four shorter peptides termed E6* (Shally et al., 1996). Of the four E6* sequences only E6*I is entirely contained within the E6 ORF, while the others continue into the E7 and E2/E4 region (Doorbar et al., 1990; Snijders et al., 1992). These alternatively spliced transcripts are the most abundant in HPV containing cell lines (Doorbar et al., 1990; Shirasawa et al., 1991), and a peptide of the expected size corresponding to E6*I could be detected in tumours induced in nude mice, but not in cell lines derived from cervical tumours (Schneider-Gadicke et al., 1988). Initial studies led to the hypothesis that the E6* encoding transcripts were by-products of the splicing event leading to the efficient expression of full length E7 (Sedman et al., 1991; Snijders et al., 1992). More recent experiments have however demonstrated that E7 is equally expressed

from either bi-cistronic or spliced transcripts, therefore raising the question of the role of the E6* encoded peptides (Stacey et al., 1995; Yamada et al., 1995). Studies on the E6*I encoded peptide have indeed demonstrated that it may be functional, having the ability to trans-activate the autologous P97 promoter, while the opposite effect was obtained using full-length E6, pointing at opposing functions of these two proteins (Shirasawa et al., 1994). Further work reported that the HPV-18 E6*I protein can also interact with the full-length E6 as well as with the E6-AP ubiquitin ligase. As a result of these interactions, E6*I can inhibit E6-mediated degradation of p53 (Pim et al., 1997) and interestingly, the binding to full length E6 may inhibit E6-directed p53 degradation (Pim and Banks, 1999). Moreover, ectopic expression of the HPV-18 E6*I protein was found to have an antiproliferative effect in cell lines derived from cervical tumours (Pim et al., 1997), resulting in p53-dependent apoptosis (Pim and Banks, 1999).

E6 structure and binding motifs.

The E6 proteins are small proteins of approximately 18 kDa and have a conserved predicted structure containing four metal-binding motifs, Cys-X-X-Cys, forming two zinc fingers (Cole and Danos, 1987; Barbosa et al., 1989), whose integrity is essential for nearly all the interactions with cellular proteins and for correct localization (Kanda et al., 1991; Sherman and Schlegel, 1996). The E6 structure is highly conserved among different viral types, both high- and low-risk (**Fig. 6**), with a hydrophilic N-terminal domain preceding the zinc fingers, which are separated by a hydrophobic region. Amino acids between 120 and 151 contain an apparent bipartite nuclear localization signal (Sherman and Schlegel, 1996), that was very recently demonstrated to be a true NLS in the case of HPV-16 E6 (Le Roux and Moroianu, 2003). As can be seen from **Fig. 7**, this region is not conserved in the E6

HPV E6 PROTEIN



E6 interacting cellular protein	High Risk	Low risk	Cellular localisation
P300/CBP	Yes	Yes	nucleus
E6AP	Yes	No	Nucleus/cytoplasm
p53	Yes	Yes	nucleus
paxillin	Yes	Yes	Plasma membrane, focal adhesions
C-myc	Yes	Yes	nucleus
Mcm7	Yes	Yes	Nucleus/cytoplasm
Bak	Yes	Yes	mitochondria
Magi-1, -2, -3	Yes	No	Plasma membane, tight junctions
MUPP-1	Yes	No	Plasma membane, tight junctions
Dlg	Yes	No	Adherens junctions, cytoplasm, nucleus

Fig. 6 The HPV E6 protein. Schematic representation of E6 showing The two zinc fingers, together with the regions involved in interactions with some of its cellular binding partners.
On the right panel are summarized some of E6 binding partners, they are localised throughout the cell and with the exception of the PDZ containing proteins they are all bound both by low- and high-risk E6 Proteins.

proteins derived from different HPV types.

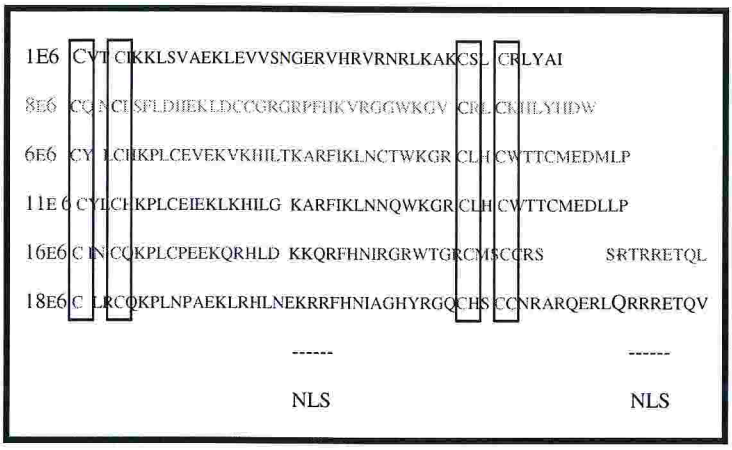


Fig.7. Sequence alignment of the C-terminal portion of the E6 proteins from cutaneous low-risk HPV-1 and high-risk HPV-8, mucosal low-risk HPV-6 and HPV-11, and mucosal high-risk HPV-16 and HPV-18. In yellow the box highlights the Cysteins (C) forming the second conserved zinc-finger. The putative Nuclear Localization Signal (NLS) regions are underlined.

At their extreme C-terminus, the high-risk mucosal viruses, share a short stretch of amino acids that encodes a domain (XT/SXV), able to bind PDZ-containing proteins (Doyle et al., 1996; Songyang et al., 1997). PDZs are structured domains of 80-90 amino acids which share eight segments of secondary structure, six β-strands, and two α-helices. The E6 "consensus PDZ-binding sequence" (XS/TXV), binds in a groove between the β B strand and the α B helix and the β A– β B connecting loop contains the sequence Gly-Leu-Gly-Phe, after which PDZ domains were originally named as GLGF repeats. These amino acids turn out to play an important functional role in binding the C-terminal carboxylate group of

the PDZ-binding-sequence containing proteins (Doyle et al., 1996). PDZ binding motifs, like the one present on E6, are present also on other viral oncoproteins such as the HTLV-1 Tax protein and the Adenovirus 9 E4-ORF1 protein, suggesting key common targets mediated by this short stretch of amino acids (Lee et al., 1997). It is important to mention, however, that these viral proteins bind only specific PDZ domains within the target proteins, suggesting that other amino acids within the PDZ domain mediate this specificity (Kiyono et al., 1997; Thomas et al., 2001; Thomas et al., 2002). The study of this subset of interactions, although extremely important to understand the later stages of malignant progression is not relevant for the discussion of my thesis and will not be described further (see(Mantovani and Banks, 2001) for review).

Another subset of proteins are bound by E6 through the so called L2G box (Tong and Howley, 1997; Chen et al., 1998; Elston et al., 1998; Kuhne and Banks, 1998). This interaction involves an α-helical motif, and is present in many cellular regulatory proteins that may represent members of common growth control pathways perturbed by E6 (Kuhne and Banks review). A third group of E6 partners is probably present, where different, yet unidentified, interaction modules may link the two partners; an example of this last group are the transcriptional co-activators p300 and CBP (Patel et al., 1999; Zimmermann et al., 1999).

E6 localization.

The lack of good antibodies against the E6 proteins has led, in the past, to highly controversial reports as to the localization of the protein. Initial studies, using an E6 protein expressed in insect cells identified the E6 protein both in nuclear and membrane fractions (Grossman et al., 1989), and this has been recently reconfirmed by a work showing that E6

localises mainly in the non-nuclear membranes and in the nuclear matrix fraction of the cell (Daniels et al., 1998). A slightly different result was obtained by immunofluorescence staining in monkey COS-1 cells, where E6 was mostly nuclear, while a Zinc-finger mutant (Cys-66) was localised in the cytoplasmic (Kanda et al., 1991).

Attempts to examine the localization of the endogenous proteins have also produced discordant results with E6 being detected in the cytoplasm in HPV positive HeLa, CaSki and SiHa HPV cells by one group (Liang et al., 1993), and in the nucleus, associated with condensed chromatin, as well as with nuclear ribonucleoproteic ultrastructures and with some ribosomal areas in the cytoplasm, by another group (Masson et al., 2003).

Although the presence of a putative NLS was recognized early on from the secondary structure (Sherman and Schlegel, 1996), it was only recently experimentally demonstrated to be real, suggesting that at least under certain conditions, E6 can enter the nucleus by an active mechanism (Le Roux and Moroianu, 2003). In this paper a GST-E6 fusion protein was used to visualise the nuclear import in digitonin-permeabilized cells, showing that sequences between amino acids 121 and 124 of HPV-16 E6 code for a NLS. Interistingly, E6 is able to enter the nucleus through multiple pathways, using both a classical Kap α2 β1-mediated pathway, as well as by binding directly to both Kap β1 and Kap β2 (Le Roux and Moroianu, 2003). The mechanism by which E6 localization is controlled however still remains to be investigated.

E6 functions:

E6 prevention of cellular senescence.

The E2 protein is able to represses the viral promoter when present at high concentrations (Bouvard et al., 1994), and this results in direct repression of the E6 and E7 oncogenes.

Recent papers have used this ability of E2 to study the effect of reintroduction of the viral protein into cervical cancer derived cell lines, demonstrating that E2 induces cellular senescence by elevating the levels of the cyclin/cdk inhibitor p21(CIP) (Wells et al., 2000; DeFilippis et al., 2003). This is thought likely to occur by a reduction in E6 protein levels, subsequent reactivation of the p53 pathway, as well as reduction in telomerase activity, thus triggering both senescence and apoptosis (DeFilippis et al., 2003). The ability of E6 to induce telomerase activity was first described by McDougall and coworkers, who showed such an effect in normal human epithelial cells (Klingelhutz et al., 1996). This activity of E6 is thought to be critical for bypassing cellular senescence, it is separable from p53 degradation, but it is not sufficient to induce immortalisation (Klingelhutz et al., 1996; Kiyono et al., 1998). Interestingly it was recently demonstrated that E6 induces high levels of transcription of the catalytic subunit of the telomerase, hTERT, by binding to c-myc and to a proximal E-box on the telomerase promoter (Veldman et al., 2003). The interaction between c-myc and E6 had indeed previously been described, and according to Gross-Mesilaty and co-workers E6 can induce the degradation of c-myc through the proteasome pathway (Gross-Mesilaty et al., 1998). This apparent discrepancy between these studies may be resolved by considering the double nature of c-myc, being able to induce both proliferation and differentiation, and E6 may need to direct its degradation at some stages of the viral life cylce, while it may need to cooperate with it at others.

E6 effects on cell cycle and differentiation

Cells expressing high-risk E6 have an altered G1 checkpoint, due to p53 inactivation and lack of p21 induction (Slebos et al., 1995). They do not respond to p53-induced growth arrest following DNA damage (Kessis et al., 1993), and additionally have a perturbed G2

checkpoint, due to both p53 degradation (Scheffner et al., 1990; Werness et al., 1990) and to direct alteration of the cyclinB/cdc2 complex by E6 (Xiong et al., 1996).

E6 proteins of both benign and oncogenic HPV types have also been found to directly interact with hMcm7, a component of the DNA replication licensing complex. This interaction results in mcm7 destabilisation, and this might be important for viral genome replication (Kuhne and Banks, 1998; Kukimoto et al., 1998), but it could potentially lead to chromosomal abnormalities that are often found in human cells expressing E6s of oncogenic HPVs.

As already mentioned, one of the characteristic features of HPV is the dependency upon cellular differentiation, and the E6 protein is specifically involved in modifying terminal differentiation of epithelial cells, which normally leads to keratinisation and subsequent cell death. HPV-16 E6 is able to impair cell differentiation in the ocular lens of transgenic mice, avoiding cell denucleation via a p53-independent mechanism (Pan and Griep, 1994; Pan and Griep, 1995; Nguyen et al., 2002b). Moreover, expression of HPV-16 E6 causes a largely delayed differentiation program in K14-E6 (keratin-E6) transgenic mice, and this effect is also largely p53 independent, being observed also in a p53 null background (Song et al., 1999). The abilty of E6 to block cellular differentiation was also verified *in vitro*, where it can block human keratinocyte differentiation induced by serum and calcium (Sherman and Schlegel, 1996). Interestingly this feature did not correlate with either binding to p53 or to E6BP (E6 Binding Protein) (Sherman et al., 2002). E6BP/ERC-55 is a putative calcium binding protein localised in the endoplasmic reticulum (Weis et al., 1994), that is targeted by both E6 and E6AP (Chen et al., 1995). The effects of this interaction might have to do with perturbation of the normal keratinocyte differentiation schedule induced by calcium, however this possibility still needs further investigation.

E6 interaction with p53.

P53 regulation by localization

There is strong evidence linking the inactivation of p53 function to the development of many human malignancies (Vogelstein, 1990). The p53 protein is a bona fide tumour suppressor (Eliyahu et al., 1989; Finlay et al., 1989) and can act both as a transcriptional activator (Farmer et al., 1992; Funk et al., 1992; Kern et al., 1992) and repressor (Ginsberg et al., 1991). p53 is a key regulator of cellular homeostasis, possibly not required for normal cell proliferation, but essential in response to cytotoxic and genotoxic insults. Normally, in fact, the p53 protein is present at a low concentration, but several types of DNA damage can activate it, including DNA double-strand breaks produced by γ-irradiation and the presence of DNA repair intermediates after UV-irradiation or chemical damage. In addition to DNA damage, hypoxia is able to stimulate p53 levels and activate the p53 protein, as well as a reduced ribonucleoside triphosphate/ monophosphate ratio (Levine, 1997). p53, as expected from its role, is highly regulated in the cell, both at the post-translational level, as well as at the localization level. The p53 protein shuttles between the cytoplasm and the nucleus, in a cell cycle-dependent manner (Shaulsky et al., 1990), and its nuclear accumulation is required for its activity (Shaulsky et al., 1991). Unsurprisingly therefore, many tumour types involve p53 sequestration in the cytoplasm (Lu et al., 2000), and a similar strategy is adopted by DNA tumor viral proteins such as the adenoviral E1B 55kD protein and the hepatitis B virus HBx protein (Elmore et al., 1997; Wienzek et al., 2000) in order to inactivate p53 functions. HPV E6 has also been shown to affect p53 localization, and this aspect will discussed below. The mechanism by which p53 enters the nucleus has been extensively studied. It has a bipartite NLS present within the

tetramerization domain between amino acid residues 305-306 and 316-325 (Liang and Clarke, 1999). Mutagenesis in this region induces the synthesis of a p53 protein, which is almost completely cytoplasmic (Dang and Lee, 1989; Shaulsky et al., 1990). More recently, a nuclear export signal (NES) has also been mapped between residues 340-351. This NES has been shown to be exposed and functional in the dimeric protein, but to be buried in the oligomerization domain when the tetramer is formed. Therefore a model has been proposed in which p53 shuttles in and out of the nucleus when not in the tetrameric form (Stommel et al., 1999).

P53 regulation by degradation

Degradation of a protein via the ubiquitin-proteasome pathway involves an enzymatic cascade that results in the covalent binding of multiple ubiquitin chains to lysine residues on the target protein (Ciechanover et al., 2000). This process can be mediated by several complexes within the cell (Banks et al., 2003), but all of them are directed to the 26S proteasome, that selectively degrades polyubiquitinated proteins to small peptides (Voges et al., 1999). All systems proceed by first activating ubiquitin (E1 enzyme), that is then transferred to an ubiquitin-conjugating enzyme (E2 enzyme). The final step is mediated by an E3 enzyme that provides specificity to the reaction. Under normal growth conditions, p53 is turned over by the ubiquitin-proteasome system via the mdm2 RING finger ligase (Maki et al., 1996; Ashcroft and Vousden, 1999; Oren, 1999). Interestingly while p53 is found to be mutated in the majority of human cancers, no mutation has been detected in HPV containing cells (Crook et al., 1991; Scheffner et al., 1991; Wrede et al., 1991). This latter observation correlates with the major oncogenic activity of the E6 proteins, being the

ability to interact with and target p53 for proteasome mediated degradation (Scheffner et al., 1990; Werness et al., 1990). The interaction, however, does not involve mdm2, but an alternative cellular protein that acts as an E3 ubiquitin ligase (Huibregtse et al., 1991; Huibregtse et al., 1993a; Scheffner et al., 1993). This protein has been designated E6AP (E6 Associated Protein) and interacts with E6 through an alpha helical motif (LLG box) that is conserved in other E6 targets (Huibregtse et al., 1993b; Kuhne and Banks, 1998).

E6AP belongs to a special class of E3 enzymes, called HECT domain containing ligases (Homologous to E6-AP Carboxyl-Terminus), that transfer ubiquitin to an internal Cys residue prior to its conjugation to the target. E6AP mutations in hippocampal and Purkinje neurons is linked to the development of the Angelman syndrome (AS), a severe mental retardation and coordination disorder. Several other putative E6AP substrates have been described (Kuhne and Banks, 1998; Thomas and Banks, 1998; Kumar et al., 1999; Oda et al., 1999), however their direct link to the AS phenotype remains to be demonstrated. Interestingly E6AP does not seem to be involved in the regulation of p53 levels in cells that do not contain E6 (Beer-Romero et al., 1997; Talis et al., 1998), so a switch to E6AP mediated degradation must occur only in the presence of E6 (Hengstermann et al., 2001).

Returning to the regulation of p53 by nuclear export, in both E6-E6AP and hdm2 mediated degradation, p53 seems to be directed to the proteasomes both in the cytoplasm and in the nucleus, since leptomycin B (LMB) treatment, that blocks CRM1 dependent nuclear export, results in p53 upreglation and activation, but only partially rescues p53 from degradation (Freedman and Levine, 1998). Although this is still a controversial issue, it seems that the ubiquitinylation step is required, since an mdm2 that lacks such activity, also fails to export p53 (Boyd et al., 2000). This suggests a mechanism by which the ubiquitin modification exposes a NES, making it more accessible to the CRM1 exportin, but this hypothesis has not been yet proven.

As mentioned above p53 is functionally active when in the nucleus, and to go back to the beginning of my introduction, it is more active, when concentrated in PML Oncogenic Domains (Gostissa et al., 1999; Fogal et al., 2000). In these domains, the presence of CBP/p300 provides the environment for acetylation on p53 K382, that mediate cellular senescence (Pearson et al., 2000), and HIPK2 mediates the phosphorylation on p53 S46, which contributes to p53-activated gene expression, and thus to cell cycle arrest and apoptosis.

Objectives of the study.

From the above introduction it is clear that targeting nuclear structures, highly enriched in specific regulatory proteins might be essential, for low abundant proteins such as E6 and E7, to hijack the cellular system. Having this in mind, I started to investigate the subcellular localization of these two proteins, with particular attention to the E7 and E6 proteins derived from both high- and low-risk HPVs, as well as to the HPV-18E6*I peptide. The results from the microscopy analysis then led me into studying the interaction between E6 and the PML protein. The biological meaning and the relevance of these interactions will be discussed in detail.

Results

Localization of the HPV-11 and HPV-16 E7 proteins.

Previous studies had reported a predominantly nuclear localization for the HPV-16 E7 protein (Sato et al., 1989; Greenfield et al., 1991; Smith-McCune et al., 1999), but as described in the introduction, many discrepancies were observed due to the different systems used (Smotkin and Wettstein, 1987; Fujikawa et al., 1994; Zatsepina et al., 1997). Moreover, in all of these studies the cellular localization of the low-risk HPV-11 E7 protein was not addressed. In order to investigate the comparative pattern of expression of the two E7 proteins I used HA-tagged versions of both HPV-11 E7 and HPV-16 E7 (**Figure 8**), using a previously cloned construct of pCDNA3-HA16E7 available in the lab, and cloning 11 E7 by standard PCR method into the same HA-PCDNA3 backbone.

The choice of cell line was also an important issue, and I decided to use U2-OS (human osteosarcoma cells), since they have a morphology that is optimal for immunofluorescence purposes. They are both p53 and pRb positive, and are easily transfectable with a high efficiency, allowing examination of a large number of E7 expressing cells in each experiment. This cell line will be the standard cell line used throughout my thesis.

Cells were transfected with plasmids expressing HA-tagged HPV-11 E7 and HA-tagged HPV-16 E7. After 24h cells were fixed, stained with an anti HA polyclonal antibody (Y-11, SantaCruz), and patterns of expression assessed using confocal microscopy. The results obtained are shown in **Figure 9**. In agreement with previous studies, the HPV-16 E7 protein exhibits a largely nuclear pattern of expression with nucleolar exclusion. Similarly, HPV-11 E7 is also largely confined to the nucleus. It is also clear however, that the low-risk E7 protein has regions of concentration within the nucleus, with the presence of clear

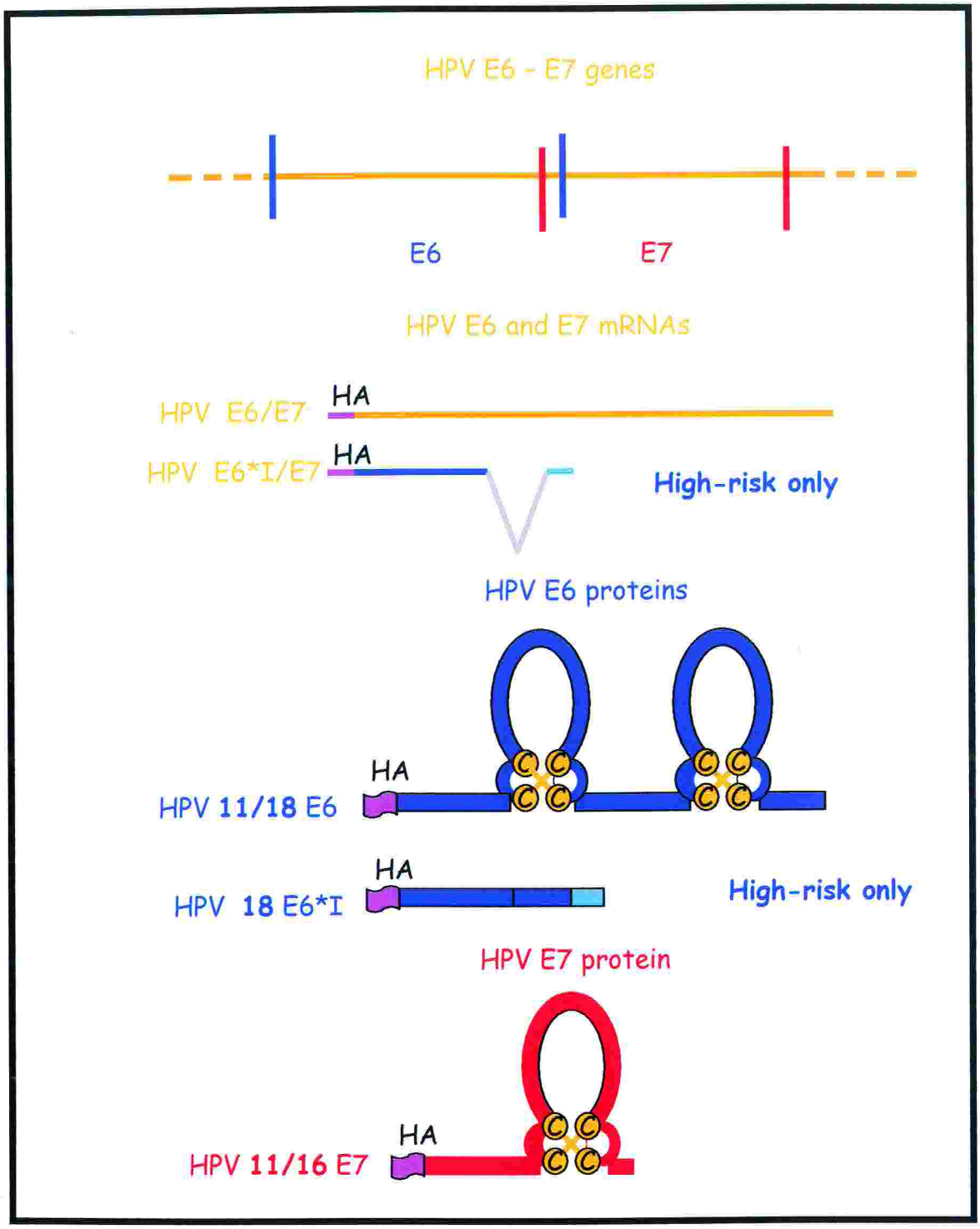


Fig. 8 Schematic representation of the constructs used in the study. HA tagged versions of HPV-11 E6 and HPV-18 E6 as well as HPV-11 E7 and HPV-16 E7 were cloned into GWI and PCDNA3 vectors respectively.

dot like structures. The experiment was also performed in HaCaT keratinocytes, with similar results, showing that localization was not cell type specific (**Figure 9B**). The nature of these structures was immediately striking since a very similar pattern of expression had been observed for proteins that localise to PML Oncogenic Domains (PODs) (Sternsdorf et al., 1997a). Therefore I proceeded to investigate whether the two E7 proteins were indeed localising to PODs. Cells were again transfected with the HPV-11 and HPV-16 E7 expression plasmids and after 24h the cells were fixed and stained for HA-E7. In addition, the cells were also stained for PML as well as for SUMO-1 (Sternsdorf et al., 1997b). The results obtained are shown in **Figure 9 C.** Again HPV-11 E7 shows nuclear localization, with a distinct punctuate pattern of expression. It is also clear from the co-staining with either anti-PML or anti-SUMO-1 antibodies, that the HPV-11 E7 protein perfectly co-localizes with PODs and that these are somewhat increased in size and altered in morphology in the presence of E7. In contrast, in the case of HPV-16 E7, it is clear that although the protein is predominantly nuclear, it does not co-localize with either PML or SUMO-1. At this stage, however, I cannot exclude whether or not 16E7 can be recruited into PODs under certain conditions. These results demonstrate a significant difference in the precise patterns of expression of the HPV-11 and HPV-16 E7 proteins within the nucleus, with HPV-11 E7 locating to POD structures, whereas the HPV-16 E7 protein does not.

Localization of the HPV-11 and HPV-18 E6 proteins.

Regulation of HPV-18 E6 nuclear import.

In order to study the localization of the HPV-18 E6 protein, I used the same approach as for E7, that is, cloning an HA-tag onto its N-terminus in the pGWI expression vector. The

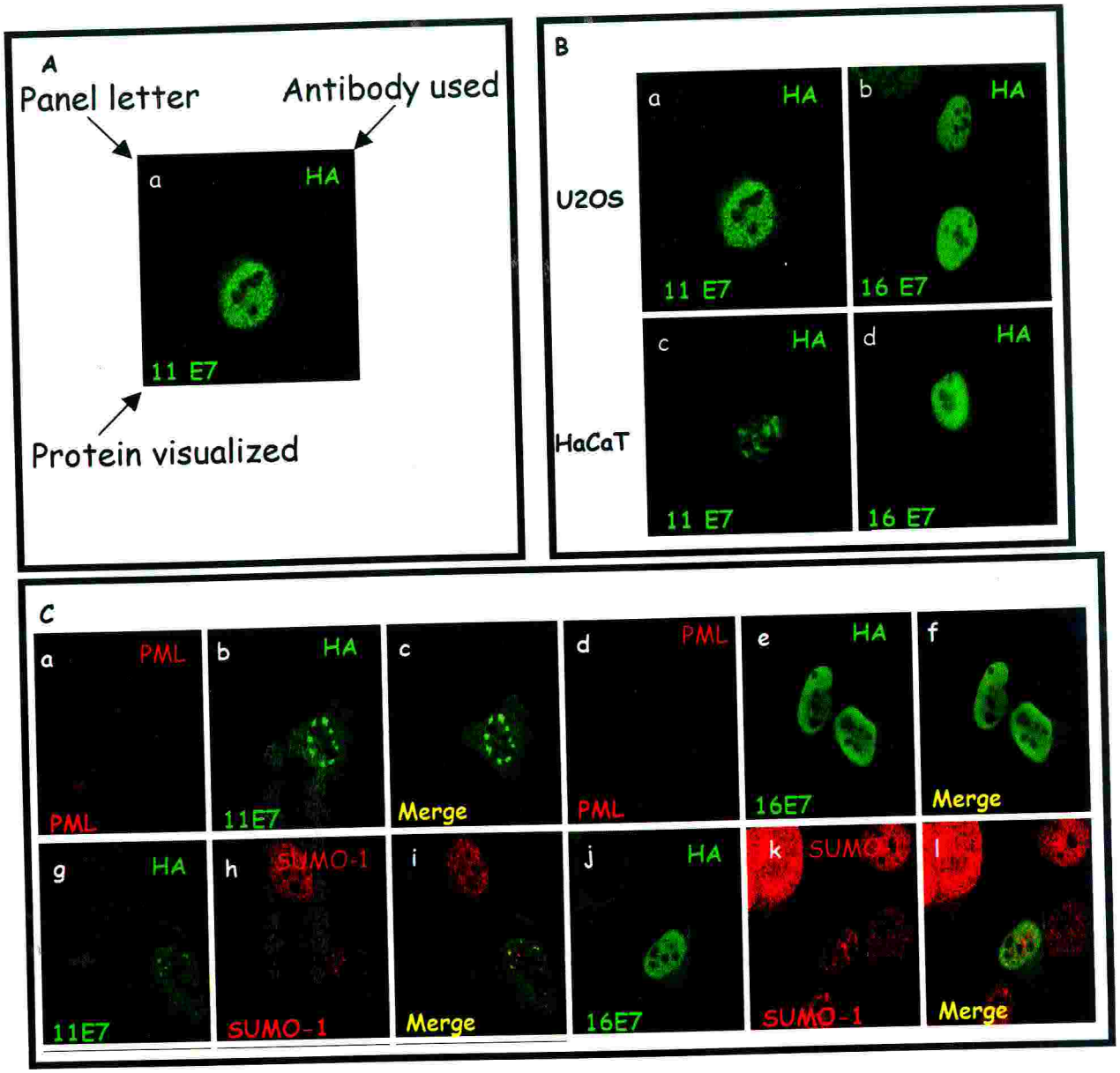


Fig. 9A Legend of the information available for all the immunofluorescence figures of this thesis. TOP LEFT: panel letter; TOP RIGHT: antibody used; BOTTOM LEFT: protein visualized
Fig. 9B HA-E7 localisation in transiently transfected U2OS cells. U2OS cells are fixed in PFA 3%, permeabilized with Triton X-100 and stained for HA-11E7 (a, c), and HA-16E7 (b, d) using an anti HA rabbit polyclonal antibody (Y-11, Santa Cruz).
Fig.9C HA-E7 localisation in transiently transfected U2OS cells. U2OS cells are fixed in PFA 3%, permeabilized with Triton X-100 and double stained for HA-11E7 and PML (a-c) and HA-16E7 and PML (d-f) or for HA-11E7 and SUMO-1 (g-i) and HA-16E7 and SUMO-1 (j-l) using an anti HA rabbit polyclonal antibody (Y-11, Santa Cruz).

resulting construct expresses not only the full length E6 protein, but also the first of the four E6* proteins, since both splice donor and acceptor sites lie within the open reading frame of HPV-18 E6 (**Figure 8**) (Doorbar et al., 1990; Snijders et al., 1992). This allowed me to monitor the localization of both full-length and E6*I. In order to differentiate between the two proteins, I mutated the HPV-18 E6 construct at the splice donor site, as previously reported for HPV-16 E6 (Sedman et al., 1991), obtaining a construct that will not splice (HA-18 E6 NS) and hence does not produce any E6* product **(Figure 10 and Figure 12A)** This construct was tested for its functionality and found to be able to degrade p53 *in vivo* (**Figure 10 B**). The localization of the full length E6 was then analysed by transient transfection in U2OS cells followed by immunostaining with a HA specific antibody. The result is shown in **Figure 11**, panel A (1), and **Figure12** panel B (c). As shown, the protein clearly accumulates within the nucleus in clear dot like structures, but it is also present in the cytoplasm, where it concentrates in aggregates. In the attempt to identify the nature of these cytoplasmic structures I also co-stained U2-OS cells for cytochrome c, since E6 has been demonstrated to associate with mitochondrial proteins such as Bak (Thomas and Banks, 1998). The results presented in **Figure 11** panel **C** and **Figure 12** panel **C** (m-o) clearly show that the confocality between the two proteins is only partial, suggesting that E6 may target multiple substructures within the cytoplasm.

One of the questions that I wanted to address next was how the nuclear entry of E6 is regulated. As mentioned in the introduction, small proteins can freely diffuse in and out of the nucleus (Rout and Aitchison, 2001; Vasu and Forbes, 2001; Pante and Kann, 2002), so one might expect this to be true also in the case of E6. High-risk E6 proteins, and in particular HPV-18 E6, have however two clear stretches of basic residues that were identified as a putative, bipartite Nuclear Localization Signal (NLS) (Sherman and Schlegel, 1996), thus posing the question as to their functionality. I mutated these two NLS

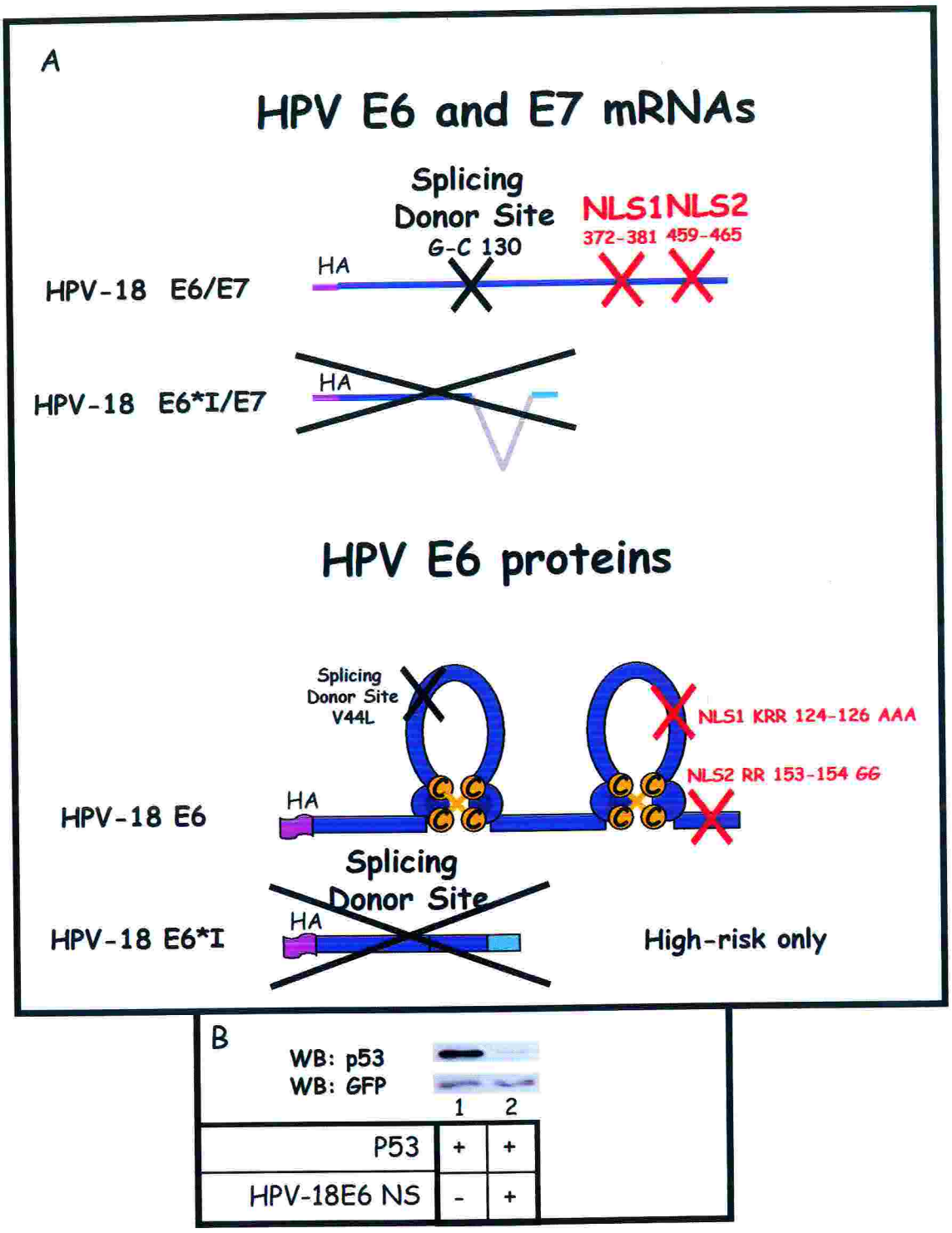


Fig. 10A Schematic representation of the mutants used in the study. **Splice Donor Site**: G to C mutation at the splice donor site (position 130 from ATG); **NLS1**: KRR at position 124-126 to AAA, and **NLS2**: RR at position 153-154 to GG.

Fig. 10B p53 degradation by full length E6 protein (NS Splicing Donor Site mutant). SaoS-2 Cells were transfected with PCDNA3-p53 and HA-18 E6 NS (1) or empty vector (2). GFP expressing vector (Clontech) was used as a control to monitor the efficiency of transfection.

by using the "Genetailor site directed mutagenesis system" (Invitrogen), and substituted the KRR at position 124-126 with AAA (NLS1 mutant), and the RR at position 153-154 with GG (NLS2 mutant).

Both mutant expressing constructs were then transfected into U2OS cells followed by immunostaining with a HA specific antibody. The results in **Figure 11** panels 2-3 clearly show that these two mutants fail to enter into the nucleus as efficiently as the wild type, and tend to accumulate in the cytoplasm where they partially co-localise with the mitochondria (**Figure 11** panel **C**). It is important to note however that a portion of the overexpressed protein is still nonetheless able to go into the nucleus, and to accumulate in dot like structures, suggesting that there are alternative pathways, other thatn the active importin-mediated one, by which E6 can enter the nucleus. The protein expression levels were also checked by standard western blot techniques and showed similar levels of expression of the three proteins (**Figure 11** panel **B**).

HPV-18 E6 and E6*I subcellular localization.

In order to differentiate between the localization of the full length E6 and the E6*I product, I then compared, both by western blotting and by immunofluorescence the expression and localization of the HA-18 E6 cDNA, HA-18 E6 NS and HA-18 E6*I. The expression of the three constructs was verified by western blot analysis: as expected the NS mutant expressed only the full length 18 E6 product (**Figure 12A-3**), while the E6*I product was only expressing the lower molecular weight product (**Figure 12A-2**), as opposed to the HPV-18 E6 cDNA that was expressing both (**Figure 12A-1**). In order to assess the pattern of localization of the three proteins, U2OS cells were transfected and after 24 hours E6 was detected using an anti HA antibody (Y-11, Santa Cruz). The results obtained are shown in

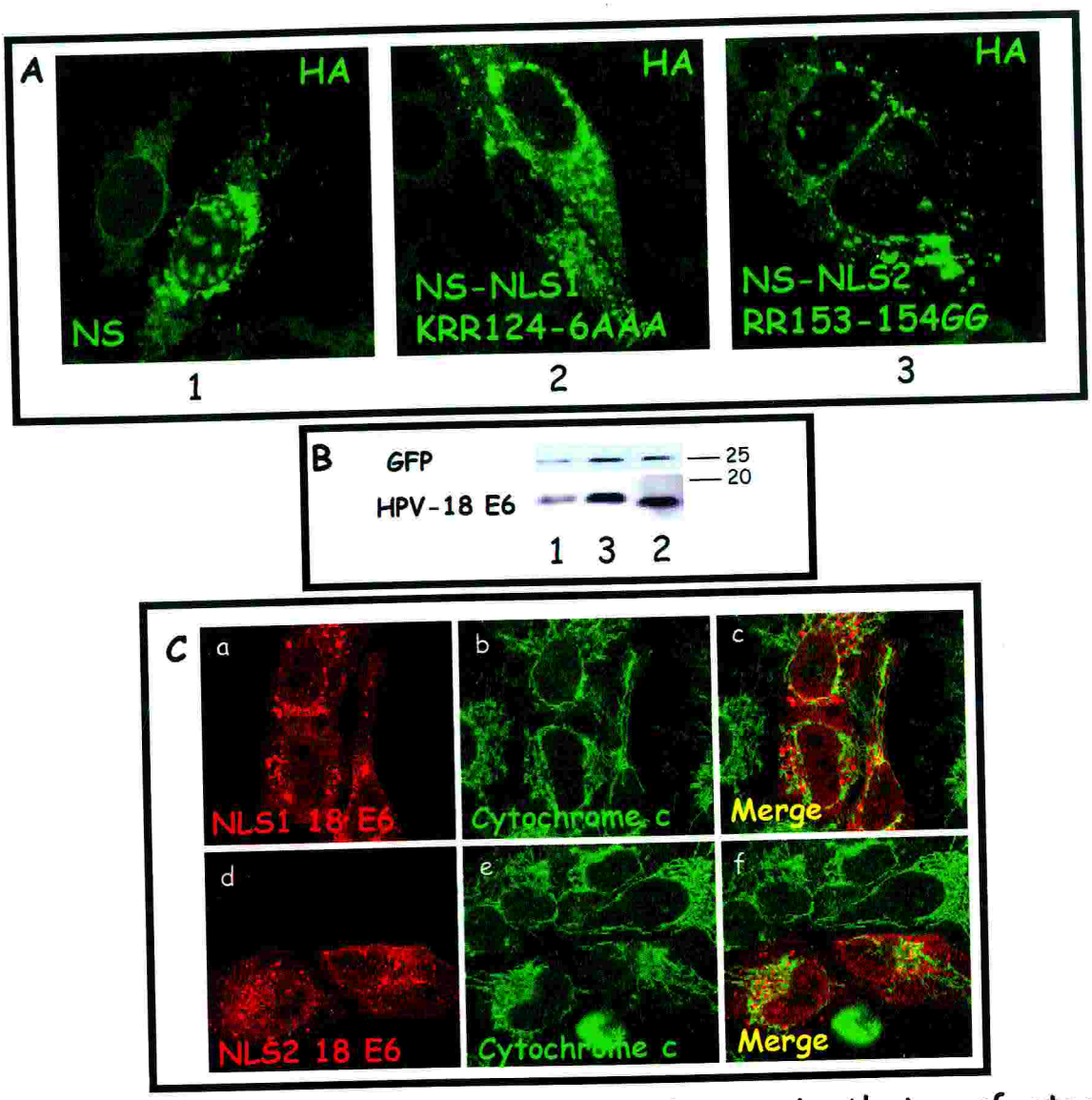


Fig. 11 Panel A. HA-18E6 localisation in transiently transfected U2OS cells. U2OS cells were fixed in 3% PFA, permeabilized with Triton X-100 and stained for HA-18E6 NS (1), HA-18E6 NS-NLS1 KRR124-6AAA (2) and HA-18E6 NS-NLS2 RR153-154GG (3) using an anti HA rabbit polyclonal antibody (Y-11, Santa Cruz).
Panel B. HA-18 E6 expression levels were assessed by western bloting using an anti-HA rat monoclonal (3F-10, Roche), GFP was used as an internal control to monitor the efficiency of transfection.
Panel C. HA-18 E6 cytoplasmic structures are only partially confocal with mitochondrial cytochrome c. U2OS cells were fixed as previously described and stained for HA-18E6 NS-NLS1 (a-c), or HA-18E6 NS-NLS2 (d-f) using an anti HA mouse monoclonal antibody (Roche) and cytochrome c using an anti cytochrome c rabbit polyclonal antibody (H-104, Santa Cruz).

Fig. 12 B. As can be seen the full length E6 protein (E6 NS) localises both in the nucleus (90% of cells) and in the cytoplasm (10% of cells) in discrete dots. The use of a construct encompassing the entire HPV-18 E6 cDNA leads to the efficient expression of both the full length E6 and E6*I and gives rise to an intermediate and diffuse pattern of expression. E6*I alone also gives a diffused pattern of expression with nuclear and cytoplasmic staining being visible. Unlike the full length protein, it does not appear to concentrate in dot-like structures (**Figure 12 B**). In order to better characterize the cellular localization of these three constructs I also stained the cells for DNA (Propidium Iodide) in order to highlight the nuclei, and for cytocrome c in order to stain for the mitochondria. As shown in **Figure 12 C**, the full length 18 E6 protein is mainly nuclear (p-r), and when present in the cytoplasm is only partially confocal with the mitochondria (m-o).

HPV-18 E6*I over-expression increases p53 levels in HPV containing cells.

Our group had previously demonstrated that HPV-18 E6*I expression in CaSKi cells had an antiproliferative effect, in contrast to the lack of effect in cells which were HPV-negative (Pim et al., 1997). Although this effect appeared to be mediated by apoptosis, the mechanism by which it was induced was not fully elucidated, thus, in order to investigate further the way by which E6*I exerts such activity, I expressed E6*I in CaSki cells (HPV+), and analysed the effect upon the levels of expression of three known target proteins, p53, Scribble and hDlg. The results obtained are shown in **Figure 13A** (immunofluorescence) and **Figure 13B** (western blot). As can be seen, E6*I expression results in a dramatic increase in p53 levels when compared with cells transfected with the control vector, expressing EGFP (compare Fig.13A a-c with d-f and Fig.13B lane 2 with

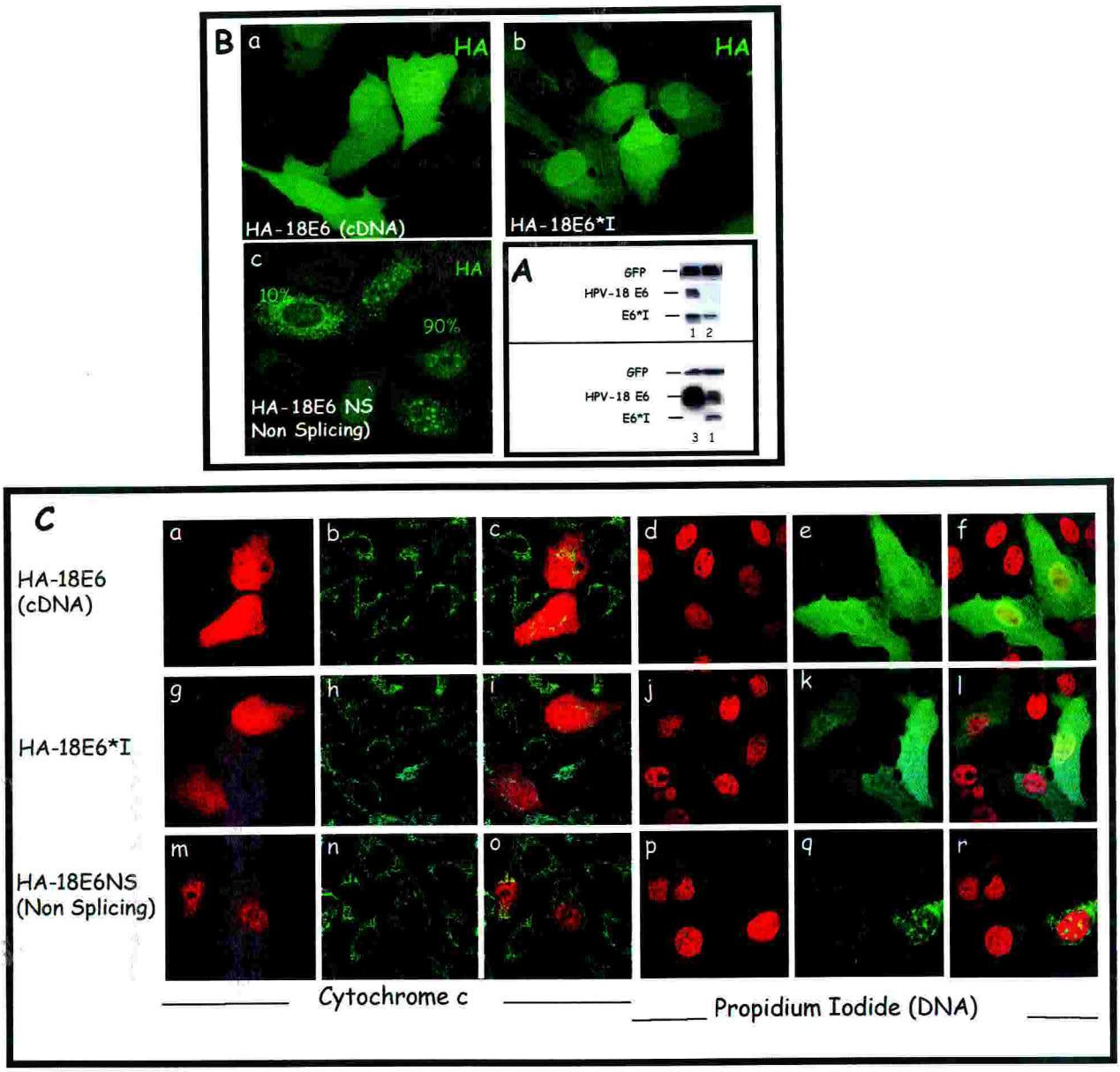


Fig. 12 Panel A. Western Blot analysis of the wild type HA-18 E6 (1) and HA-18 E6*I (2) constructs (upper panel) and the HA-18 E6 NS (3) and wild type HA-18 E6 (1) constructs (lower panel).
Panel B. Immunofluorescence analysis of transiently transfected HA-18 E6 (a), HA-18 E6*I (b) and HA-18 E6 NS (c) in U2-OS cells. Cells were fixed after 24 h in PBS/3%PFA and stained with an anti-HA antibody (Y-11, Santa Cruz).
Panel C. Immunofluorescence analysis of transiently transfected HA-18 E6 (a-f), HA-18 E6*I (g-l) and HA-18 E6 NS (m-r) in U2-OS cells. Cells were fixed after 24 h in PBS/3%PFA and double stained for HA-E6 and Cytochrome c (a-c, g-I and m-o) or DNA (propidium iodide in d-f, j-l and p-r)

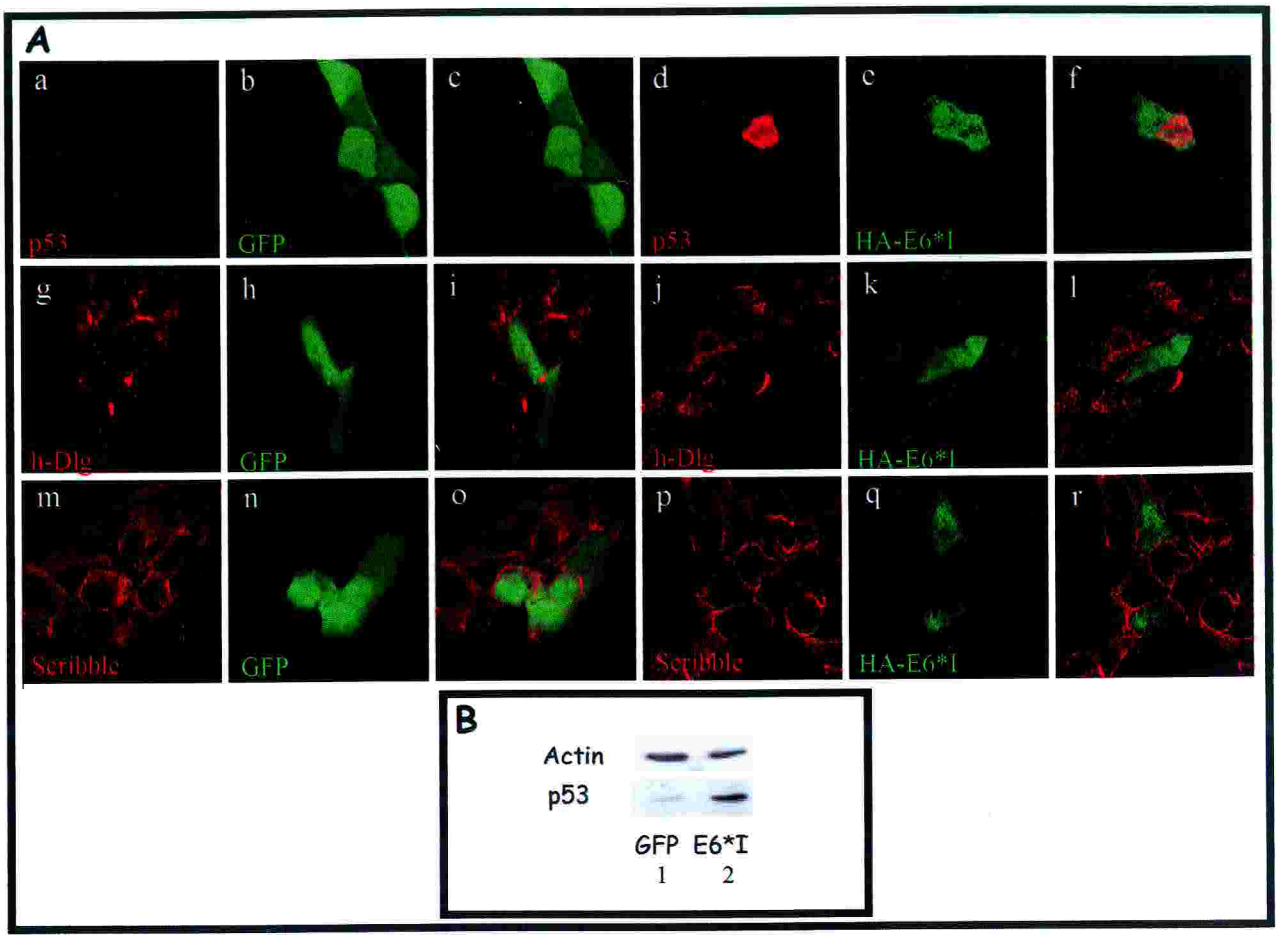


Fig. 13 Panel A. p53 upregulation in CaSki cells, following HPV-18 E6*I overexpression. CaSki cells were transfected with HA-18 E6*I or EGFP expression constructs. Cells were fixed and stained after 10 hours with an anti-HA antibody (primary: Y-11, Santa Cruz; secondary: fluorescein-green anti-rabbit, Molecular Probes) and a mouse anti-p53 antibody (a-f)(primary: DO-1, Santa Cruz; secondary: rhodamine-red anti-mouse, Molecular Probes); mouse anti-hDlg antibody (g-l)(primary: 2D11, Santa Cruz; secondary: rhodomamine-red anti-mouse, Molecular Probes) or goat anti-Scribble antibody (m-r)(primary: C-20, Santa Cruz; secondary: rhodomamine-red anti-goat, Alexa). **Panel B.** Western Blot analysis of p53 levels of expression in CaSki cells following GFP or E6*I overexpression. Lane1: cells were transfected with GFP-expression vector, Lane2: cells were transfected with HA-E6*I expression vector. Actin level is used as a loading control.

1). However there was no apparent change in the levels of expression of Scrible or hDlg (compare Fig.13A g-i to j-l and m-o to p-r). This results suggest that the antiproliferative effects of E6*I in CaSKi cells are mediated via the p53 pathway.

HPV-18 E6 and E6*I ratios vary during the cell cycle.

HPV replication is intimately linked to keratinocyte differentiation and this leads to a maintenance of the cell cycle in cells that are normally committed to terminal differentiation (Stubenrauch and Laimins, 1999). In order to study possible variations of the E6/E6*I ratio during the cell cycle and thus any regulatory functions of the E6*I peptide in this context, I transfected U2-OS cells with the HA-tagged wild type HPV-18 E6 cDNA. After 24 hours, the cells were treated with aphidicolin, a drug that induces reversible G1 arrest, for a further 24 hours, and then released from the G1 block by washing them several times with pre-warmed medium. At this time (time zero) cycling cells and G1 blocked cells were harvested. Five hours after the addition of fresh 10% FCS (Foetal Calf Serum) DMEM, cells in S phase were harvested and, 9-10 hours after time zero, cells undergoing mitosis were harvested (M1 and M2). Cells were analysed by FACS to verify the correct continuous cell cycle (**Figure 14A**) and a cyclin B1 western blot was done as a control for correct M phase progression (**Figure 14B**). Western blots were then probed for the full length and alternatively spliced form of E6. As can be seen from **Figure 14B**, the full length E6 protein does not vary during cell cycle progression. In contrast, the E6*I splice product increased progressively through the cell cycle being most abundant during G2/M.

Since I had shown that E6*I rescues p53 from E6 mediated degradation *in vivo*, I then wished to investigate whether there was a corresponding upregulation of p53 in CaSKi

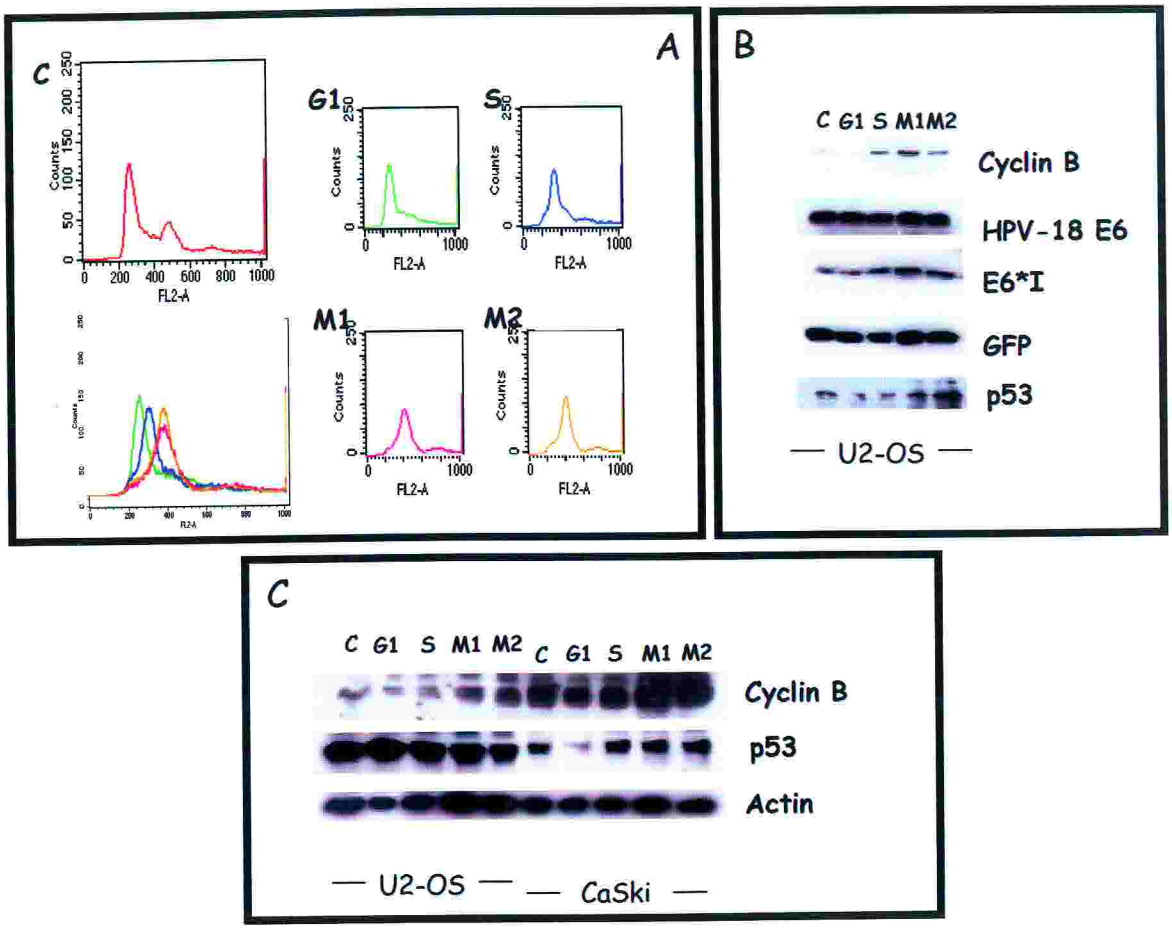


Fig. 14 Panel A. FACS analysis on transiently transfected U2-OS cells. Cells have been synchronized by a single 24h aphidicolin block and then released. C: Cycling cells; G1: time zero after 24h of aphidicolin block; S: S phase, 5 hours post-release; M1: mitosis, 9 hours post-release; M2: mitosis, 10 hours post-release.
Panel B. Western Blot analysis of HA-18 E6 expression in synchronized U2-OScells. Anti-HA (3F10) is used to detect both HA-18 E6 full length and HA-18 E6*I. Anti-p53 (DO-1 Santa Cruz) is used to detect endogenous p53. Anti-cyclin B (Santa Cruz) and anti-GFP (living color peptide, Clontech) are used to check for synchronization and for equal transfection efficiency, respectively.
Panel C. Western Blot analysis of p53 levels of expression in synchronized CaSki and U2-OS cells. Anti-p53 (DO-1, Santa Cruz) is used to detect endogenous p53. Anti-cyclin B (Santa Cruz) and anti-actin (Sigma) are used to check for synchronization and for equal loading, respectively.

cells (HPV +) during G2/M, which might be expected if E6*I levels did increase. In order to do this CaSKi cells (HPV +), and as a control U2-OS cells (HPV-), were synchronized and the levels of p53 assessed during the cell cycle, probing with a mouse monoclonal anti-p53 antibody (DO-1, Santa Cruz). The results obtained are shown in **Figure 14 C**. It is clear that whilst p53 levels are low during G1/S in CaSKi cells, there is a marked increase during G2/M. In contrast, the U2-OS HPV negative cells exhibit higher levels of p53 during G1/S. These results further support the notion that E6*I levels vary during the cell cycle, and that at its highest point of accumulation during G2/M there is also a concomitant increase in p53 protein levels. This was further confirmed by probing for p53 in synchronized U2-OS cells that had been previously transfected with an E6 expressing construct. The results are shown in **Figure 14 B** (lower panel) and, consistent with E6*I accumulation during G2/M, the p53 levels also increase accordingly.

HPV-18 E6*I regulates the levels of HPV-18 full length E6.

In order to investigate how E6*I might modulate E6 activity, we transfected U2-OS cells with the non-splicing mutant (E6NS) alone, with E6*I alone and with the two in combination. A high salt extraction (0.5 M NaCl) was then used to separate the cytoplasmic and nuclear soluble fraction from the nuclear insoluble fraction, as described in the materials and methods section. As shown in **Figure15A**, E6*I expression reduces the levels of E6 in the insoluble fraction. Interestingly the levels are restored by proteasome inhibitor treatment (50μM N-CBZ-LEU-LEU-LEU-AL MG132 in DMSO), two hours prior to harvesting. Both E6 and E6*I are stabilized by MG132 suggesting that both proteins are degraded along the proteasome pathway. A recent report has indeed demonstrated that this is true for the full length E6 protein, and unlike for E7, E6

ubiquitination does not seem to involve the N-terminus, which was blocked by the HA tag in our experiments (Kehmeier et al., 2002).

Localization of the full length HPV-11 and HPV-18 E6 proteins.

There are many conflicting reports on the cellular location of the high risk HPV E6 proteins, with expression being detected in many different locations within the cell (Grossman et al., 1989; Kanda et al., 1991; Liang et al., 1993; Sherman and Schlegel, 1996; Daniels et al., 1998; Masson et al., 2003). In contrast there have been no studies on the pattern of expression of the low risk E6 proteins. Therefore I proceeded to compare the pattern of expression of HPV-11 E6 and HPV-18 E6. U2OS cells were transfected with plasmids expressing HA tagged versions of the two proteins and cells were fixed and stained after 24hrs. I analyzed the localization of both proteins in several cell lines, namely the HPV negative U2-OS (human osteosarcoma cell line p53+, pRb+), SaOS-2 (human osteogenic fibrosarcoma cell line, p53-, pRb-), MG63 (human osteosarcoma cell line p53-, pRb+) and HaCaT (immortalized human skin keratinocytes, p53 mut, pRb+), as well as the HPV positive CaSki (human cervical epidermoid carcinoma cell line, HPV16 positive) and HeLa (human cervical carcinoma cell line, HPV18 positive). The results obtained are shown in **Figure 16** and clearly show that, similarly to HPV-18 E6, HPV-11 E6 is also largely localized to the nucleus, and also exhibits a marked punctuate pattern of expression. Since I have already found that the HPV-11 E7 protein localized to POD structures (Fig. 9C), and that the full length E6 proteins also display a similar punctuate pattern of expression, I therefore proceeded to investigate whether the E6 proteins were also localizing to PODs. Cells were thus co-stained for the endogenous PML proteins (PG-M3, Santa Cruz), and the results assessed by confocal microscopy. As can be seen from **Figure**

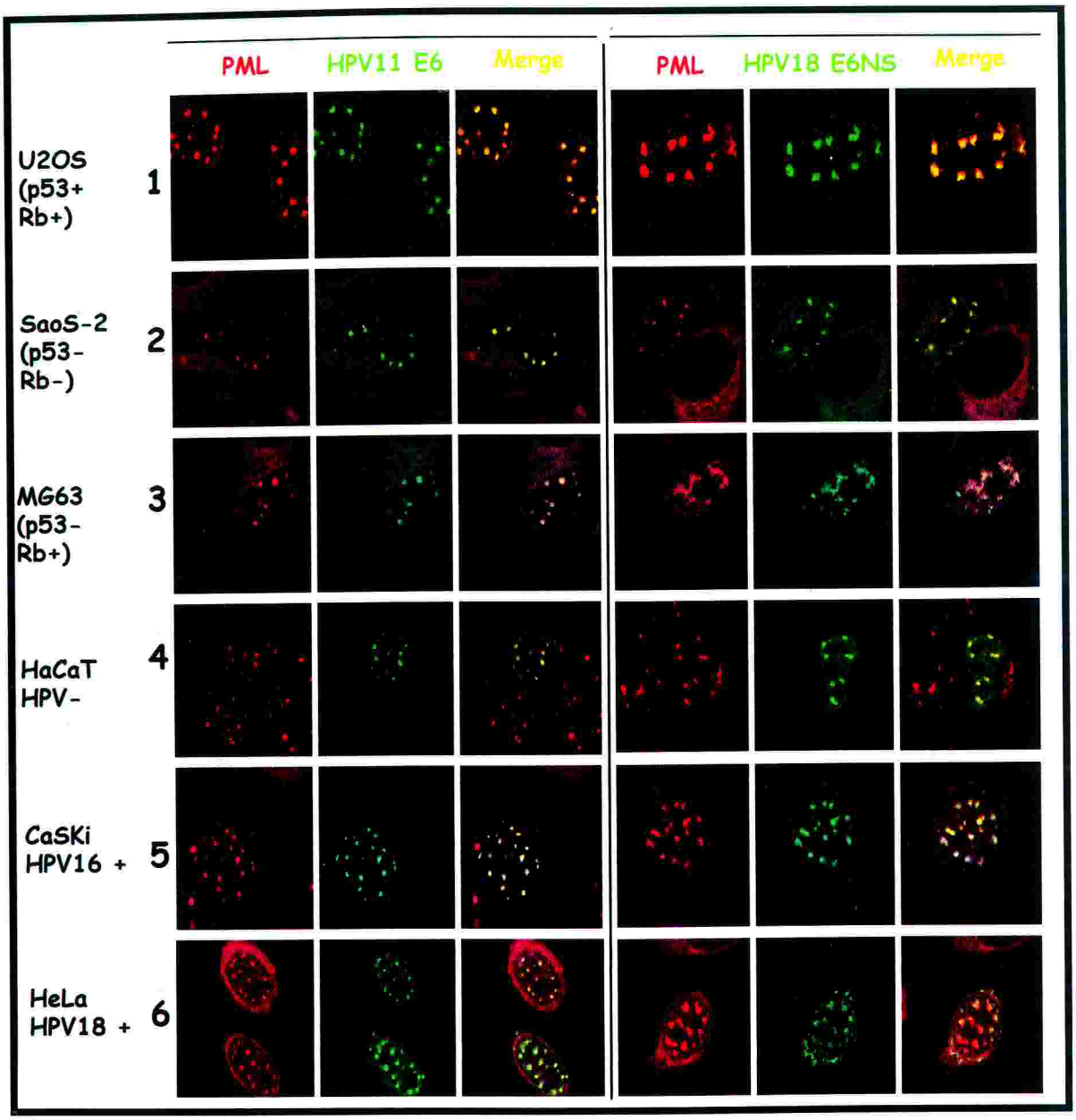


Fig. 16. Localisation of HA-tagged HPV-11 E6 and HA-tagged HPV-18 E6NS (expressing just the full length E6) in transiently transfected **U2OS** cells (lane 1), **SaoS-2** (lane 2), **MG63** (lane 3), **HaCaT** (lane 4), **CaSKi** (lane 5), and **HeLa** cells (lane 6). Cells were fixed in PBS/3%PFA, permeabilised with PBS/0.1%Triton X-100 and stained for anti-HA (green, Y-11, Santa Cruz) and for endogenous PML (red, PG-M3, Santa Cruz).

16 the E6 proteins co-localize with most of the endogenous PML protein.

HPV-11 E6 and HPV-18 E6 are confocal with PML isoforms I-IV

Previous studies have shown that there is extensive alternative splicing of the PML gene resulting in at least seven different isoforms (Jensen et al.,2001). Although the function of these different isoforms is unknown, many studies have reported isoform specific protein interactions (Fogal et al., 2000; Wu et al., 2001; Li et al., 2000; Alcalay et al., 1998), suggesting that not all forms are functionally equivalent. Therefore I was particularly interested in determining whether the HPV-11 and HPV-18 E6 proteins were targeting any specific subset of PML isoforms. In order to do this I made use of a panel of FLAG-tagged PML isoforms that are depicted schematically in **Figure 17A**. I first verified their correct expression and localization in U2-OS cells. As can be seen from **Figure 17B**, over-expression of these different isoforms all gives rise to punctuate nuclear staining, with some variation in the size, shape and number of dots depending on the particular PML isoform. To investigate whether the HPV E6 oncoproteins were being selectively targeted to specific isoforms I performed co-transfection experiments in U2-OS cells with the HA tagged HPV E6 proteins and the FLAG tagged PML isoforms. After 24 hrs the cells were fixed and stained for both sets of proteins using HA and FLAG specific antibodies to identify the specific isoforms (HA: Y-11, Santa Cruz 1:200; FLAG: M2, Sigma 1:1000). The results for HPV-11 E6 are shown in **Figure 18** and the results for HPV-18 E6 are shown in **Figure 19**. As can be see, HPV-11 E6 shows a very high degree of confocality with PML isoforms I-IV. In contrast, there is no significant degree of co-localization with PML isoforms V and VI. Similarly, HPV-18 E6 shows a very high degree of confocality with PML isoforms I-IV but not with isoforms V and VI. Therefore these results

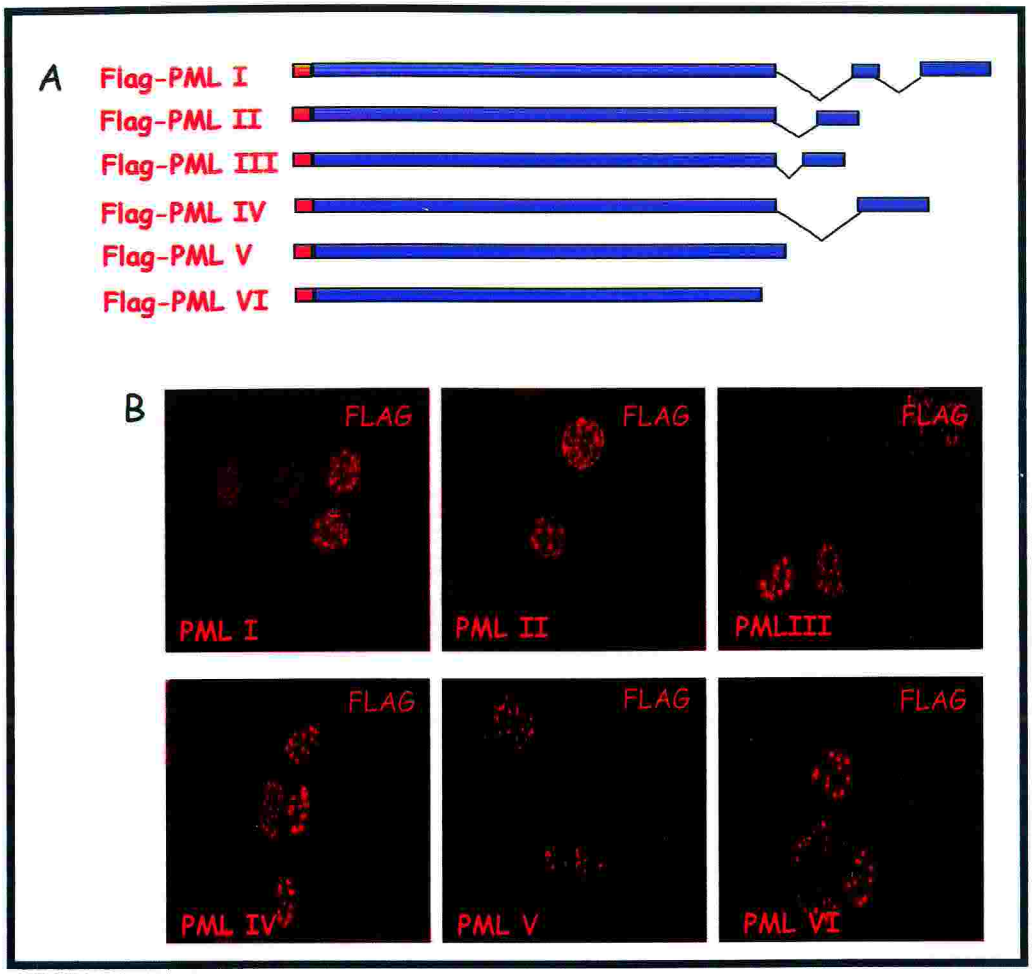


Fig. 17 Panel **A** shows a schematic representation of the different PML constructs used in the study. The PML isoforms are depicted as the alternatively spliced variant mRNAs that were cloned downstream of the FLAG tag coding sequence. Panel **B**. Localization of the different FLAG- tagged PML isoforms in transiently transfected U2-OS cells. The cells were fixed and permeabilized as previously described and the PML detected using an anti-FLAG monoclonal antibody (M2, Sigma).

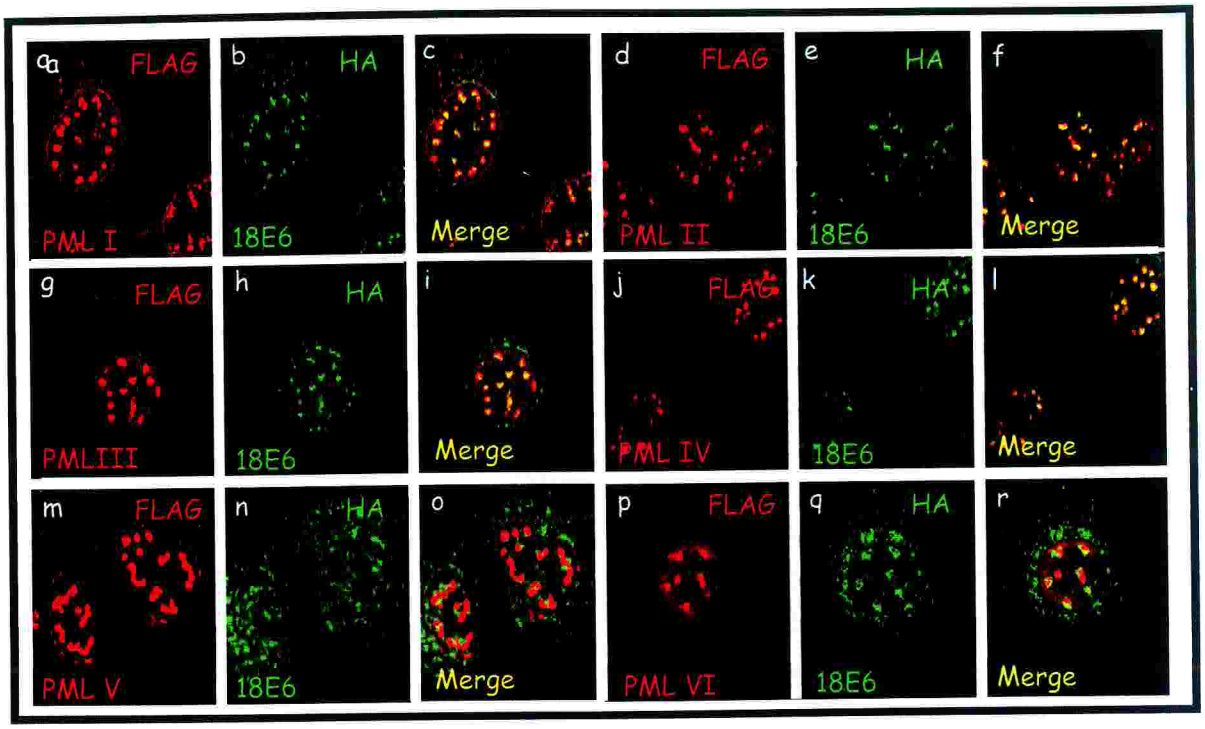


Fig. 19. Confocal analysis of the pattern of expression of HA-tagged HPV-18 E6 and the different FLAG-tagged PML isoforms in transiently transfected U2-OS cells. The cells were fixed and double stained for anti HA-18E6 (primary: Y-11, Santa Cruz; secondary: fluorescein-green anti rabbit, Molecular Probes) and anti FLAG-PML (primary: M2, Sigma; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), HPV-18 E6 alone (green) and the merged image. The PML isoforms analysed are as follows: isoform I (a-c), isoform II (d-f), isoform III (g-i), isoform IV (j-l), isoform V (m-o) and isoform VI (p-r).

demonstrate that the localization of E6 to PODs is highly specific in that only a subset of these structures are being targeted. In addition, it also shows that the particular PODs to which the two different E6 proteins are being targeted are also similar, suggesting that at this level there is a high degree of conservation of function between the low- and high-risk HPV E6 proteins.

To further charachterize E6-PML confocality and to exclude any potential artefacts from the use of the anti-FLAG antibody, I repeated the experiment, this time staining the cells with a PML specific antibody (PG-M3, Santa Cruz 1:200), which is able to detect the endogenous PML protein as well as the overexpressed one. The results obtained after transfecting cells with the constructs expressing HA-18E6 and the different isoforms, are shown in **Figure 20**. As expected, the pattern of expression is very similar to that seen in Figure 19. The only difference is that there are minor regions of confocality observed, in the presence of overexpressed isoforms V and VI, probably due to the presence of the endogenous isoforms I-IV within the PODs.

HPV E6 proteins interact with specific PML isoforms *in vitro*.

Having shown that the HPV E6 proteins are targeted to PML isoform-specific-PODs, we were then interested in determining whether the E6 proteins were capable of interacting with the respective PML proteins. In order to do this, in vitro translated ^{35}S-labelled-PML isoforms were incubated with GST-11 E6 and GST-18 E6, as well as with GST alone as a negative control for 90 mins at room temperature. The beads were then washed, and bound proteins detected following SDS-PAGE and autoradiography. The results are shown in **Figure 21A**. As can be seen, a strong interaction was obtained between both E6 proteins and PML isoforms I and IV, a somewhat weaker interaction was obtained with PML

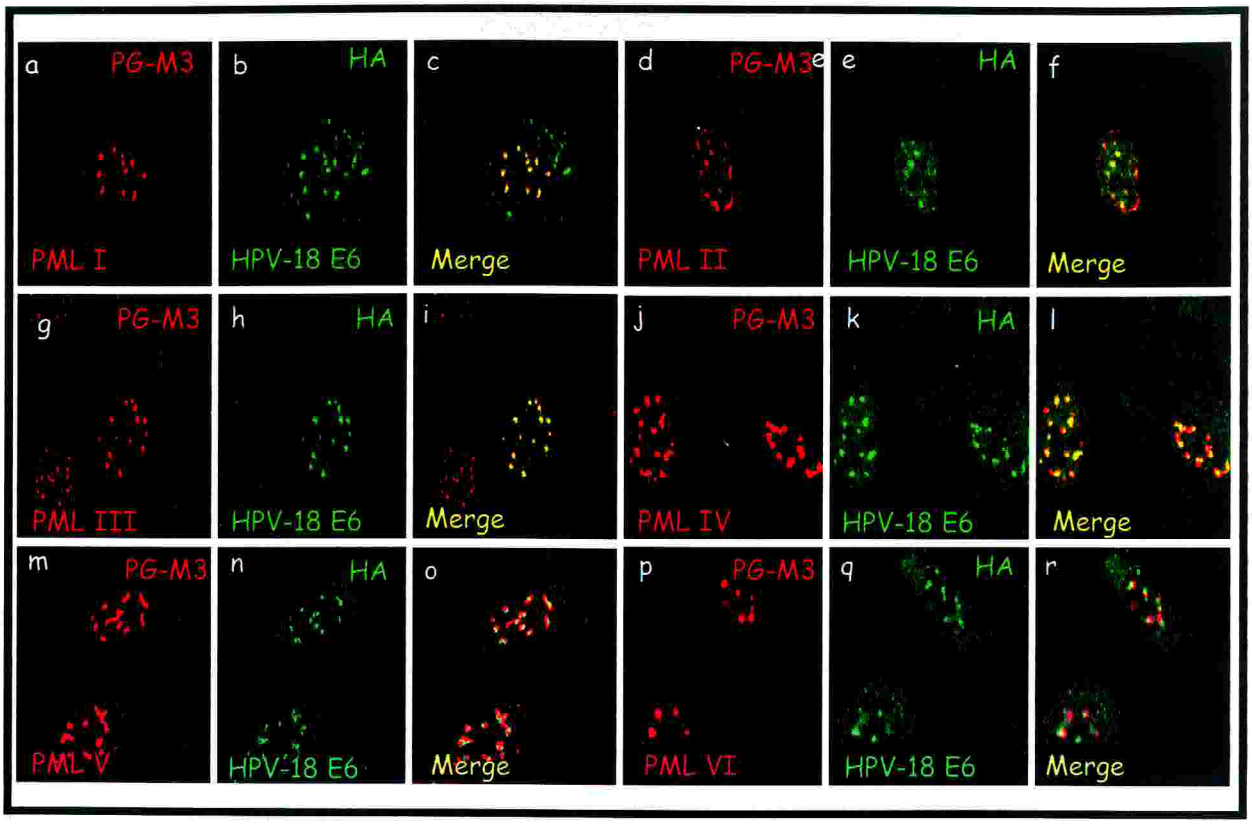


Fig. 20 Confocal analysis of the pattern of expression of HA-tagged HPV-18 E6 and the different FLAG-tagged PML isoforms in transiently transfected U2-OS cells. The cells were fixed and double stained for anti HA-18E6 (primary: Y-11, Santa Cruz; secondary: fluorescein-green anti rabbit, Molecular Probes) and anti PML (primary: PG-M3, Santa Cruz; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), HPV-18 E6 alone (green) and the merged image. The PML isoforms analysed are as follows: isoform I (a-c), isoform II (d-f), isoform III (g-i), isoform IV (j-l), isoform V (m-o) and isoform VI (p-r). The staining with the PG-M3 antibody allows detection of both endogenous and overexpressed PML in U2-OS PML+/+ cells.

isoform II, whilst no binding was seen between the E6 proteins and PML isoforms III, V and VI, nor between PML and the GST negative control. Interestingly both 11 E6 and 18 E6 bound the different PML isoforms with equal affinity. A Coomassie stain of the gel shows equal input of the GST fusion proteins (**Figure 21B**), while **Figure 21C** shows the average percentage binding, with the standard deviations from three independent experiments. Finally I also investigated whether the E7 proteins could also bind PML, since HPV-11E7 had showed a high degree of confocality with PML in immunofluorescence experiments (**Figure 9**). As can be seen from the binding assay shown in **Figure 21D,** no binding could be detected between E7 and PML IV.

The HPV E6 proteins interact with specific PML isoforms *in vivo*.

To assess if the interaction between E6 and PML was also occurring *in vivo,* I transfected U2-OS cells with the HA-tagged E6 proteins (2.5 μg) together with the FLAG tagged PML isoforms (0.5 μg). After 24hrs the cells were extracted and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were then analysed by western blotting for the presence of the HA-tagged E6 proteins and the results obtained are shown in **Figure 22A**. As can be seen there is a strong interaction between the HPV-11 E6 protein and a subset of the PML isoforms. The strongest interaction would appear to be between HPV-11 E6 and isoform I, II and IV, with a weak binding to isoform V. The highly specific nature of these interactions is highlighted by the lack of interaction between HPV-11 E6 and isoforms III and VI and by the first two lanes showing no E6 protein being pulled down in the absence of the plasmid expressing FLAG tagged PML. This data is highly in agreement with the *in vitro* interaction assays. In contrast, the HPV-18 E6 protein exhibits a markedly different pattern of association, with no clear evidence of interaction being detected with any of the

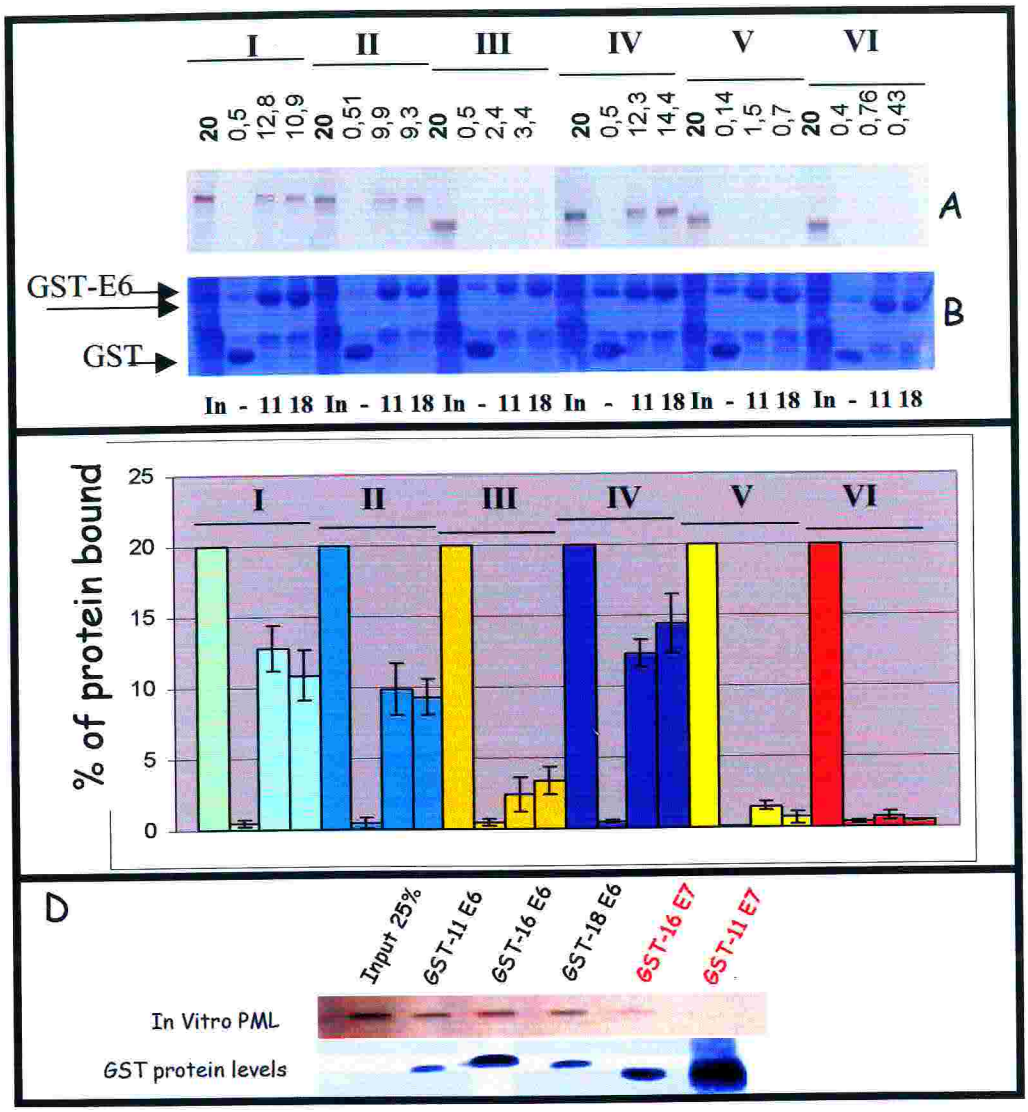


Fig. 21 The HPV E6 proteins interact with specific PML isoforms *in vitro*. **Panel A.** In vitro translated ^{35}S-labelled PML isoforms were incubated for 90 mins at room temperature with GST alone (-), GST-11E6 (**11**) and GST-18E6 (**18**). (**In**): 20%INPUT. After several washings they were subjected to SDS-PAGE and autoradiography. **Panel B.** Coomassie staining of the gel shows GST proteins inputs. **Panel C.** Quantitation of the degree of interaction between the HPV E6 proteins with the different PML isoformsThe bars represent the mean % binding from three independent experiments. The standard deviation are also shown. **Panel D.** PML does not bind in vitro to either 11E7 or 16E7. In vitro translated ^{35}S-labelled PML IV was incubated for 90 mins at room temperature with GST alone (-), GST-11E6, GST-16E6, GST-18E6, GST-16E7 and GST-11E7. After several washings they were subjected to SDS-PAGE and autoradiography. Lower panel: Coomassie staining of the gel shows GST proteins inputs.

PML isoforms. These results therefore demonstrate substantial differences between the two viral oncoproteins in how they associate with the different PML specific isoforms *in vivo*. In order to further confirm the pattern of interaction between HPV-11 E6 and the different PML isoforms the co-immunoprecipitation was repeated, but the opposite way round: pulling down the specific PML isoforms using an HA specific antibody, followed by western blot staining with a specific anti-FLAG antibody. The results are shown in **Figure 22B**. Once again the detected interactions are between PML isoforms I, II and IV and 11E6. Taken together, the *in vitro* and the *in vivo* binding data show a clear consensus of HPV-11 E6 binding to PML isoforms I, II and IV and not to PML isoforms III, V and VI. In the case of HPV-18 E6 a similar pattern is seen *in vitro*, but the lower binding affinity between PML and 18E6 *in vivo* was extremely puzzling and will be discussed later.

HPV-11 E7 and HPV-16 E7 do not co-localize with the same PML isoforms as HPV-11 E6 and HPV-18 E6.

Having found that both E6 proteins from HPV-11 and HPV-18 as well as the HPV-11 E7 protein can co-localize with PODs, I was then interested in determining whether HPV-11 E7 was targeting the same PODs structures as HPV-11E6. To investigate this I performed co-transfection experiments into U2-OS cells with the HA tagged HPV E7 plasmids and the FLAG tagged PML isoforms. After 24 hrs the cells were fixed and stained for both sets of proteins using anti-HA and anti-FLAG specific antibodies (HA: Y-11, Santa Cruz 1:200; FLAG: M2, Sigma 1:1000). The results for HPV-11 E7 are shown in **Figure 23** and the results for HPV-16 E7 are shown in **Figure 24 A**. As can be seen, HPV-11 E7 shows a very high degree of confocality with PML isoforms I, II and IV, while it shows no confocality with isoforms III, V and VI. Interestingly, unlike the HPV-E6 proteins it does

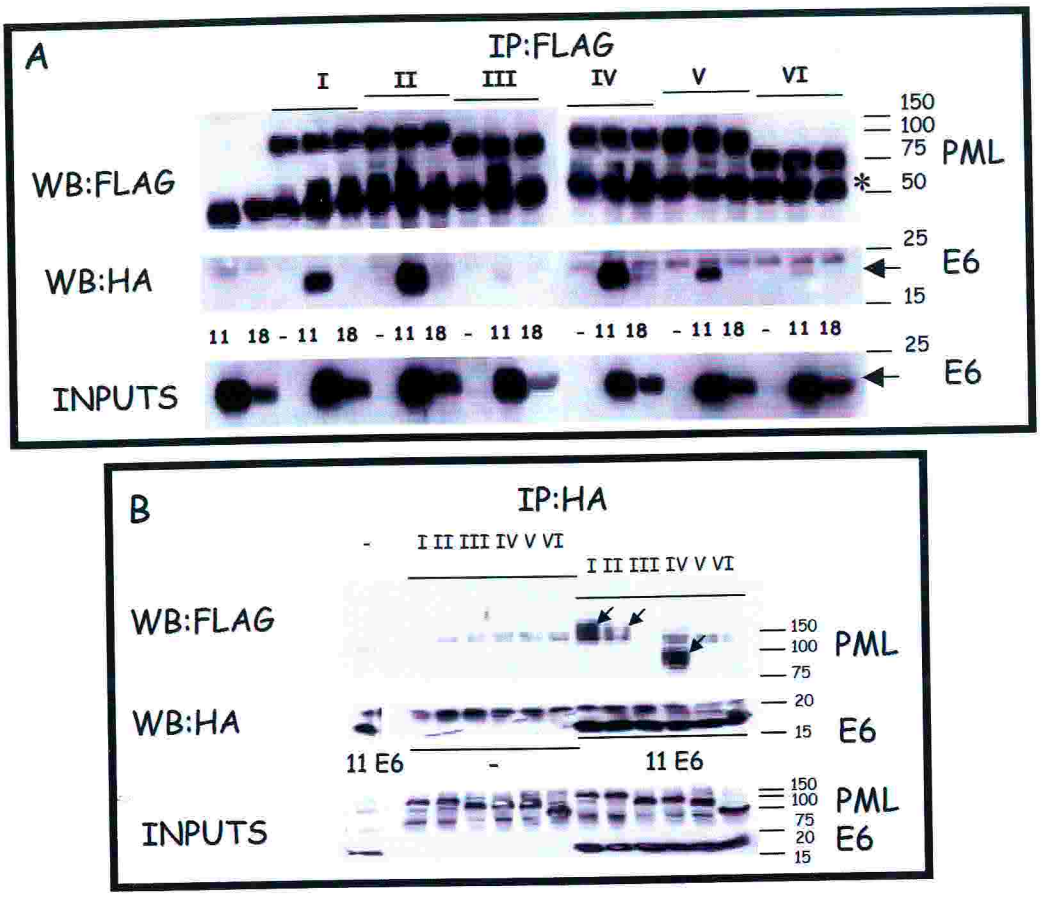


Figure 22. The HPV-11 E6 protein interacts with specific PML isoforms *in vivo*. U2-OS cells were transiently transfected with the different FLAG-tagged PML isoforms either alone or together with HPV-11E6 and HPV-18E6 expression constructs. After 24hrs the cells were harvested and the lysates were immunoprecipitated with an anti-FLAG antibody (M2)(**Panel A**) or an anti-HA antibody (3F-10)(**Panel B**). The immunocomplexes were then analysed by western blotting with an anti-HA (3F10) antibody to detect co-immunoprecitated HPV E6 proteins, or with an anti-FLAG (M2) antibody to detect the immunoprecipitated PML isoforms. Arrows indicate the respective proteins whilst *denotes the IgG heavy chains. The bottom panels show the western blot representing 10% of the total protein used in the co-immunoprecipation experiment. As can be seen there is strong co-precipitation between HPV-11 E6 and PML isoforms I, II and IV.

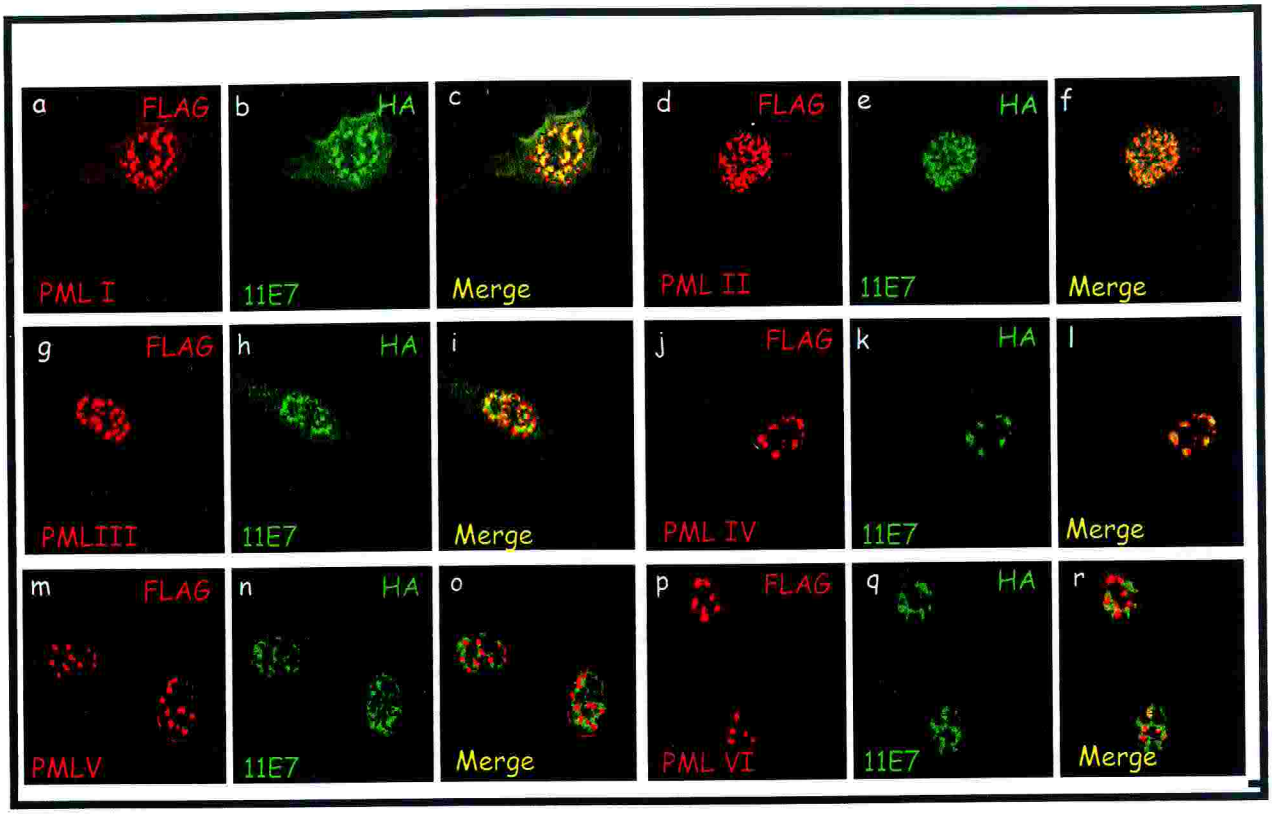


Fig. 23 HA 11E7 colocalizes with PML isoforms I, II and IV. Confocal analysis of the pattern of expression of HA-tagged HPV-11 E7 and the different FLAG-tagged PML isoforms in transiently transfected U2-OS cells. The cells were fixed and double stained for anti HA-11E7 (primary: Y-11, Santa Cruz; secondary: fluorescein-green anti rabbit, Molecular Probes) and anti FLAG-PML (primary: M2, Sigma; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), HPV-11E7 alone (green) and the merged image. The PML isoforms analysed are as follows: isoform I (a-c), isoform II (d-f), isoform III (g-i), isoform IV (j-l), isoform V (m-o) and isoform VI (p-r).

not colocalise with PML isoform III. This result provides further evidence of the specific nature of the interactions. In contrast, HPV-16 E7 showed no significant degree of co-localization with any of the PML isoforms except for isoform IV (**Figure 24 A**), and this was only observed when both proteins were over-expressed. This could be mediated by an interaction with pRb, which has been reported to bind specifically PML isoform IV (Alcalay et al., 1998), and this has been previously demonstrated in the case of E1A (Ferbeyre et al., 2000). In order to test for this possibility, I made use of an HPV-16 E7 mutated in the LXCXE pRB interacting motif. The results are shown in **Figure 24 B** and as can be seen, this mutant still co-localizes with PMLIV, suggesting that this is not a consequence of the interaction with pRb.

The interaction between the HPV E6 proteins and specific PML isoforms is not mediated by DAXX.

Recent reports have suggested that DAXX is responsible for viral protein accumulation in PODs (Ishov et al., 2002; Becker et al., 2003). DAXX interacts with Human Cytomegalovirus (HCMV) transactivator tegument protein pp71, driving its accumulation into PODs before the production of immediate-early proteins (Hofmann et al., 2002a; Ishov et al., 2002). Moreover DAXX colocalises with HPV-33 L2 in the upper strata of HPV lesions (Florin et al., 2002a; Florin et al., 2002b) and once again DAXX seems to be required for L2 accumulation in PODs (Becker et al., 2003).

To test if DAXX was similarly recruiting the E6 proteins to PODs, I first checked the co-localization of DAXX with the different PML isoforms. U2-OS cells were transfected with PCDNA3-DAXX expression construct in combination with the different FLAG-PML isoforms, and immunofluorescence staining was performed as previously described. DAXX

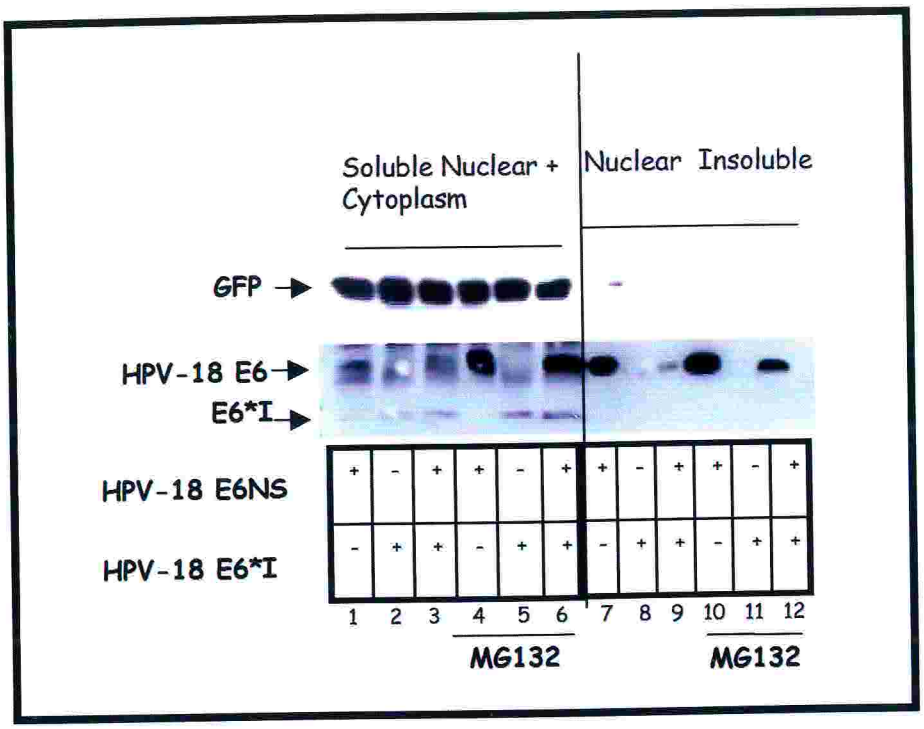


Fig. 15 Western Blot analysis of HA-18 E6NS and HPV-18 E6*I expression in U2-OS cells. Anti-HA (3F10) is used to detect both HA-18 E6NS full length and HA-18 E6*I. Anti-GFP (living color peptide, Clontech) is used to check for equal transfection efficiency.

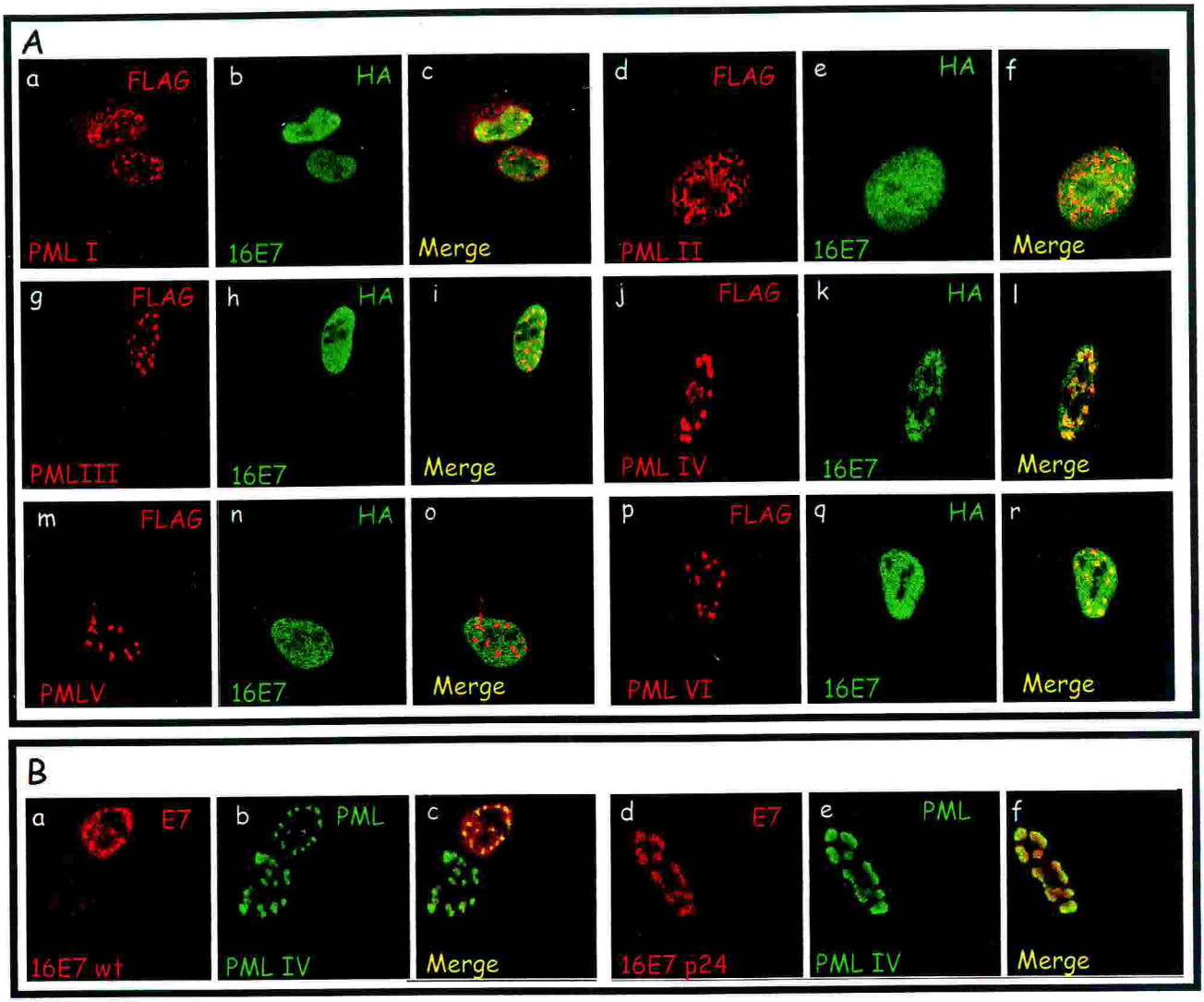


Fig. 24 Panel A. HA 16E7 colocalizes with PML isoform IV. Confocal analysis of the pattern of expression of HA-tagged HPV-16 E7 and the different FLAG-tagged PML isoforms in transiently transfected U2-OS cells. The cells were fixed and double stained for anti HA-16E7 (primary: Y-11, Santa Cruz; secondary: fluorescein-green anti rabbit, Molecular Probes) and anti FLAG-PML (primary: M2, Sigma; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), HPV-16E7 alone (green) and the merged image. The PML isoforms analysed are as follows: isoform I (a-c), isoform II (d-f), isoform III (g-i), isoform IV (j-l), isoform V (m-o) and isoform VI (p-r). **Panel B.** HA 16E7 p24 mutant colocalizes with PML isoform IV. U2-OS cells were transfected with FLAG-PMLIV and PCDNA3-wt16E7 (a-c) or PCDNA3-16 p24E7 (d-f), fixed as previously described, and double stained for 16E7wt and p24 (primary: ED-17, Santa Cruz; secondary: rhodamine-red anti mouse, Molecular Probes) and anti PML (primary: H-238, SantaCruz; secondary: fluorescein-green anti rabbit, Molecular Probes).

was found to co-localize with PML isoforms I-IV and VI. Interestingly PMLV re-localized DAXX into the nucleoplasm, with evident exclusion from PMLV-containing PODs in 60% of cells (**Figure 25**). The biological significance of this observation is at present not clear, and will be discussed below in the appropriate section. Most importantly however, DAXX co-localized with a different subset of PML isoforms when compared to the E6 proteins, thus making it unlikely that it is a mediator of E6 transport into PODs. This was further verified by a series of triple transfection experiments. U2-OS cells were transfected with PCDNA3-DAXX, HA-11 E6 (**Figure 26**) or HA-18 E6 (**Figure 27**) expression constructs, in combination with the different PML isoforms. The slides were then co-stained, in parallel, for HA-E6 and DAXX (**Figure 26A** and **Figure 27A**) (DAXX: M-112, Santa Cruz 1:200 and HA: Y-11, Santa Cruz 1:200) or for FLAG-PML and DAXX (**Figure 26B** and **Figure 27B**) (DAXX: M-112, Santa Cruz 1:200 and FLAG: M2, Sigma 1:1000). Unsurprisinlgy, DAXX and E6 maintained an independent localization showing a high degree of confocality only when PML isoforms I, II, III and IV were overexpressed. One important feature worth noting is the fact that DAXX overexpression results in HPV-11 E6 delocalization from PML IV (Figure 26B j-l), while it has no effect on HPV-18 E6 localization with the same isoform (Figure 27B j-l). This finding highlights yet another difference between the E6 proteins derived from low- and high-risk HPV types.

HPV-18 E6 targets a subset of PML IV for proteolytic degradation.

The differences between HPV-18 and HPV-11 E6 as regards to their PML interaction profiles observed *in vivo* (no or little interaction with HPV-18 E6 when compared to HPV-11 E6) as opposed to *in vitro* GST pull downs, were surprising. However previous studies have shown that the HPV-18 E6 oncoprotein can target many of its substrates for

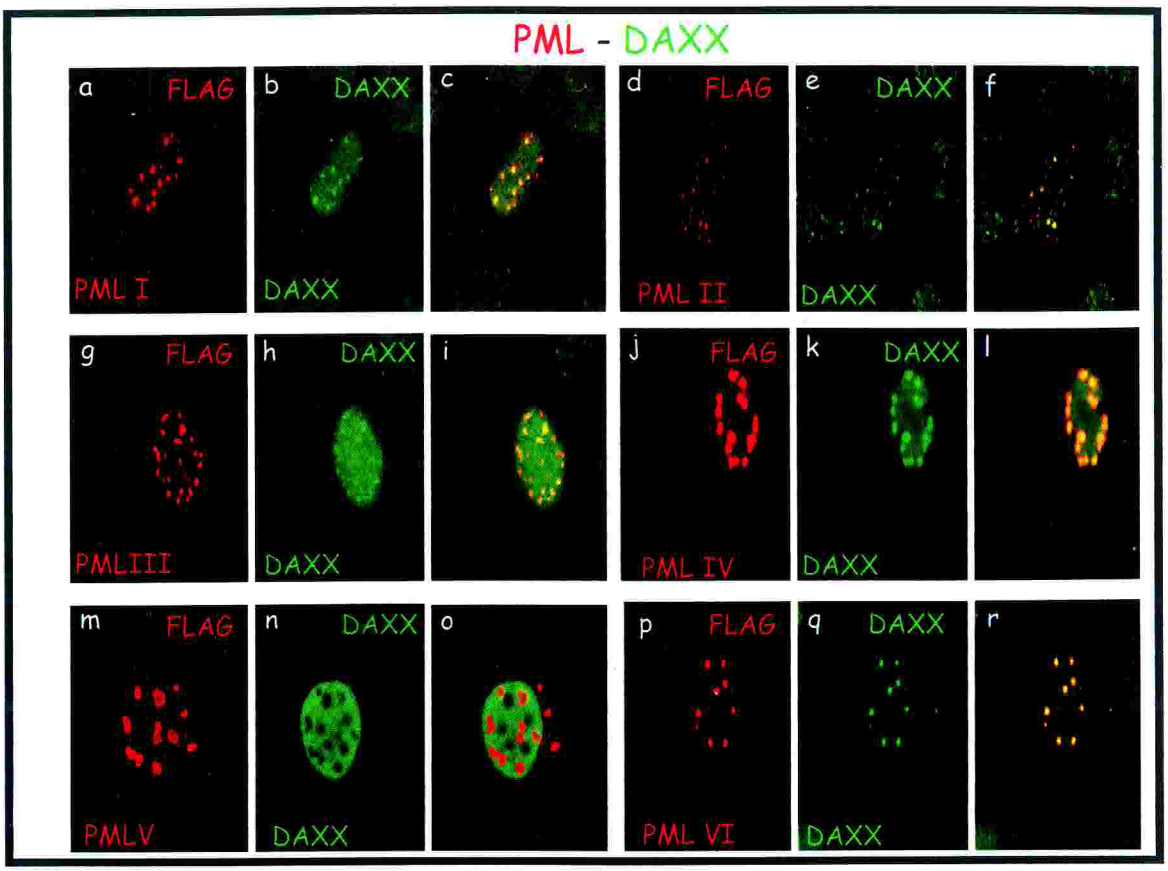
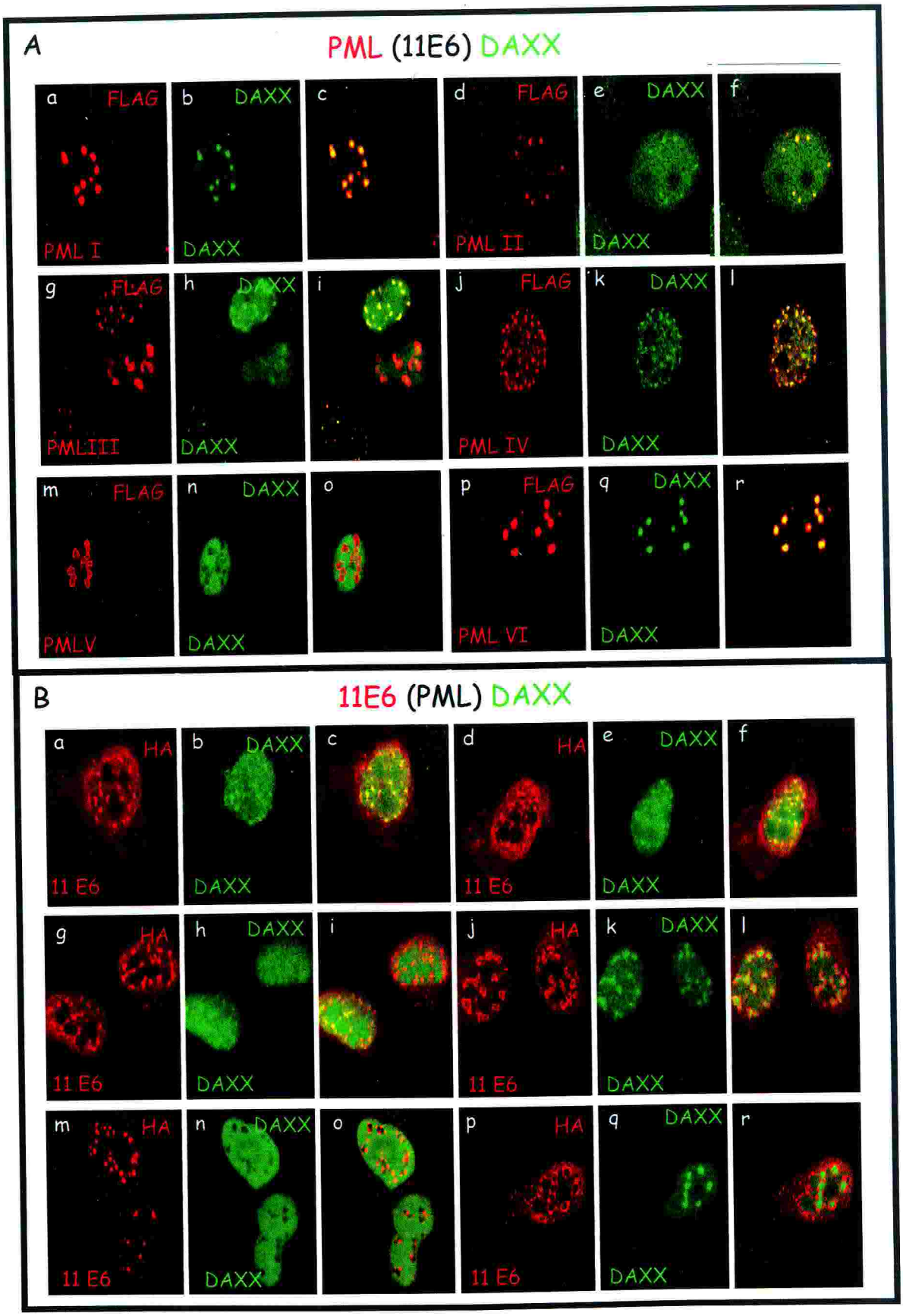


Fig. 25 DAXX colocalizes with PML isoform I-IV and VI. Confocal analysis of the pattern of expression of DAXX and the different FLAG-tagged PML isoforms in transiently transfected U2-OS cells. The cells were fixed and double stained for anti DAXX (primary: M-112, Santa Cruz; secondary: fluorescein-green anti rabbit, Molecular Probes) and anti FLAG-PML (primary: M2, Sigma; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), DAXX alone (green) and the merged image. The PML isoforms analysed are as follows: isoform I (a-c), isoform II (d-f), isoform III (g-i), isoform IV (j-l), isoform V (m-o) and isoform VI (p-r).

Fig. 26 The same experiment as in Fig.25 was repeated this time transfecting HA-11 E6, DAXX and the different FLAG-tagged PML isoforms. The cells were fixed and double stained for anti DAXX and anti FLAG-PML (**A**) and anti DAXX and anti HA (**B**).

Fig. 27 The same experiment as in Fig.25 was repeated this time transfecting HA-18 E6, DAXX and the different FLAG-tagged PML isoforms. The cells were fixed and double stained for anti DAXX and anti FLAG-PML (**A**) and anti DAXX and anti HA (**B**).


A
PML (11E6) DAXX
a FLAG
b DAXX
c
d FLAG
e DAXX
f
PML I
DAXX
PML II
DAXX
g FLAG
h DAXX
i
j FLAG
k DAXX
l
PMLIII
DAXX
PML IV
DAXX
m FLAG
n DAXX
o
p FLAG
q DAXX
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PMLV
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11 E6
DAXX
11 E6
DAXX
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r
11 E6
DAXX
11 E6
DAXX

Fig. 26

proteasome mediated degradation (Mantovani and Banks, 2001), which frequently makes detection of HPV-18 E6 bound proteins extremely difficult. To investigate this possibility further we proceeded to study the effects of HPV-11 and HPV-18 E6 upon the steady state levels of PML IV. We also used PML VI as a negative control since this isoform showed no binding to either of the E6 proteins. Cells were transfected with the constructs expressing the HA tagged E6 proteins together with those expressing the FLAG tagged PML isoforms. After 24 hrs the cells were treated for 2 hrs with the proteasome inhibitor MG132 (CBZ) in order to rescue any proteins that were being degraded at the proteasome. Cells were then extracted and divided into soluble and insoluble fractions and the presence of the PML protein detected by western blotting. The results obtained are shown in **Figure 28**. As can be seen, HPV-18 E6 induces a dramatic decrease in the quantity of the insoluble form of PML isoform IV (compare lanes 1 and 3), and this is rescued by treatment with CBZ (compare lanes 3 and 4), suggesting that it is being actively degraded at the proteasome. Interestingly, HPV-18 E6 had no effect upon the levels of expression of the soluble form of PML IV nor on the PML isoform VI, suggesting that its ability to target a subset of PML IV protein for degradation is highly specific. In contrast, HPV-11 E6 appears to have little or no effect on the steady state levels of any of the PML isoforms. One last thing that is worth mentioning is that in the presence of PMLIV, HPV-18 E6 is also more soluble and abundant in this fraction, suggesting a possible mechanism involving binding and relocalization.

HPV E6 proteins inhibit the ability of PML IV to induce cellular senescence

PML IV has been recently shown to display a specific ability to induce cellular senescence

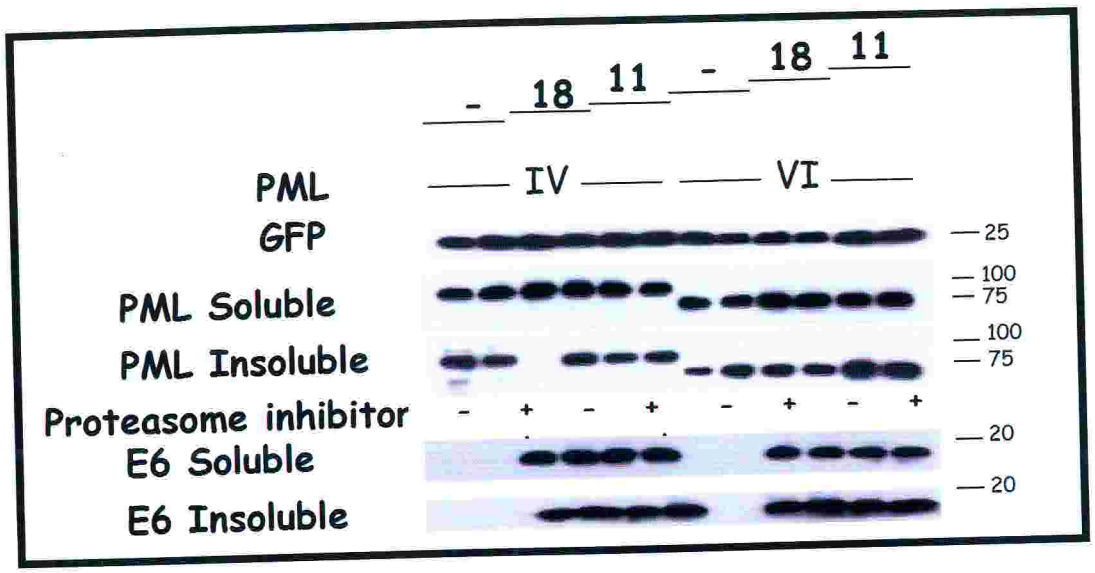

Fig. 28 The HPV-18 E6 protein targets a fraction of PML isoform IV for proteasome mediated degradation. U2-OS cells were transiently transfected with the FLAG-tagged PML isoforms IV and VI either alone or in combination with HA-tagged HPV-11 E6 and HPV-18 E6 proteins. After 24hrs the cells were harvested and a high salt (0.5M NaCl) extraction was performed to extract soluble material (PML soluble) whilst the remainder was termed insoluble (PML insoluble). The two different fractions were then analysed for steady state levels of the two different PML isoforms by western blotting with an anti-FLAG antibody (M2). The proteasome inhibitor MG132 (CBZ) was added 2 hours prior to harvesting (+) in half of the transfections to determine whether the PML isoforms were being subjected to proteasome-mediated degradation. The upper panel shows a western blot for anti GFP that was used as a control for transfection efficiency. Lane 3 shows a clear reduction in the protein levels of insoluble PML isoform IV in the presence of HPV-18 E6, which is rescued by addition of MG132 (lane 4).

in primary fibroblasts, while the other PML isoforms do not exert such activity (Alcalay et al., 1998; Fogal et al., 2000; Bischof et al., 2002). Since previous studies have shown that blocking E6 expression in HeLa cells can result in replicative senescence (Goodwin et al., 2000; Wells et al., 2000) I was interested in investigating whether the interaction between the HPV E6 proteins and PML IV might affect the ability of PML IV to induce senescence. In order to do this I established an assay for inducing cellular senescence in primary Baby Rat Kidney (BRK) cells. The cells were transfected with plasmids expressing PML IV or PML VI together with plasmids expressing HPV-11, HPV-18 E6 and a mutant of HPV-18 E6 that is negative for p53 degradation (18 E6ΔA (Pim et al., 1994)). After 12 days of selection with G418 the cells were fixed and senescent cells determined by staining for acidic β-galactosidase expression, a marker for senescence (Dimri et al, 1995). The results obtained are shown in **Figure 29** where it can be seen that approximately 12% of cells were senescent in the presence of PML IV, which is comparable to the levels obtained with the positive control of EJ-ras alone (Bischof et al., 2002). In agreement with previous studies, PML VI had no effect upon the number of senescent cells. Most interestingly however, both HPV-11 and HPV-18 E6 ΔA were both as effective as HPV-18 E6 in reducing the number of PML IV induced senescent cells to almost background levels. These results demonstrate, that although HPV-11 and HPV-18 E6 may target PML IV through different mechanisms, their net effect is the same with respect to their ability to overcome PML IV induced cellular senescence and, based on the results with the 18E6 ΔA mutant, this seems to be largely p53-independent.

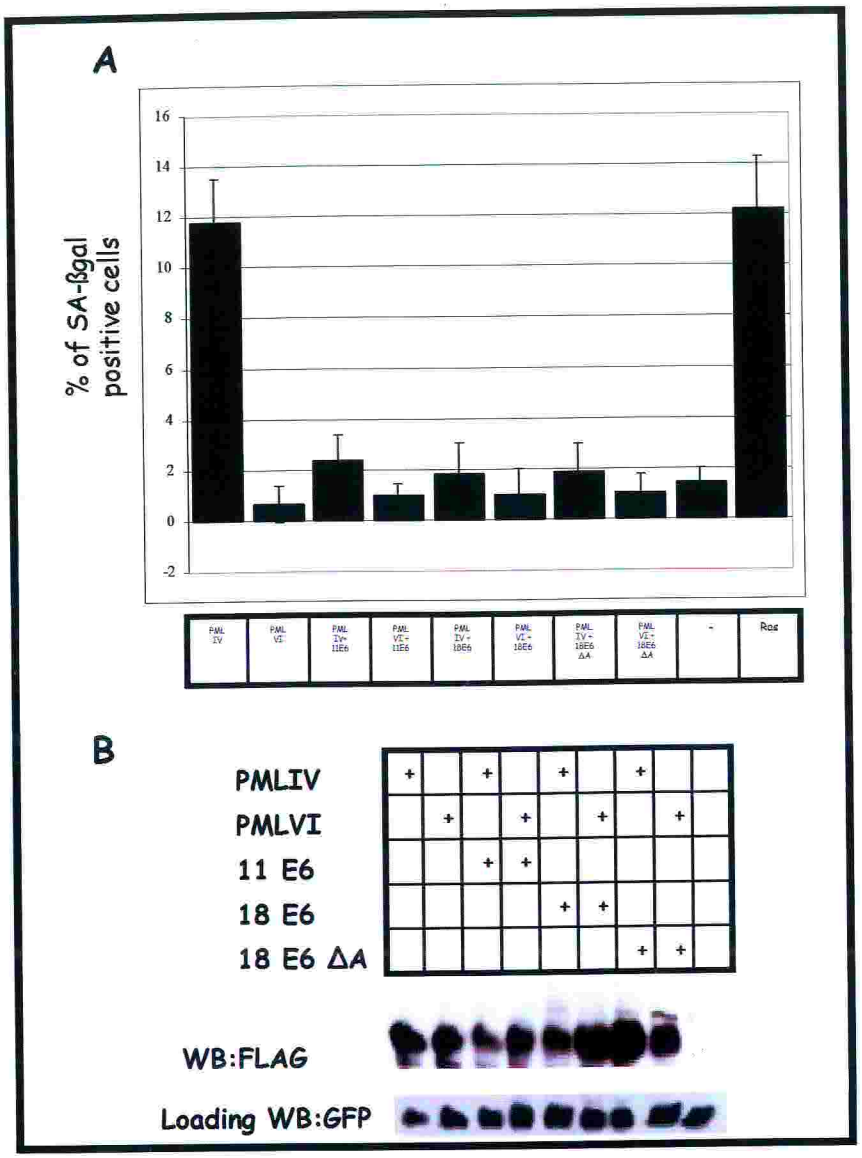

Fig. 29 A. HPV E6 inhibits PML IV induced cellular senescence. Primary BRK cells were transfected with PML IV, PMLVI or EJ-ras as a positive control, together with HPV-11 and HPV-18 E6 expression plasmids. After 12 days under G418 selection the remaining cells were fixed and stained for Senescence Associated-β-gal to detect senescent cells. The number of blue cells was then determined by microscopic visualization. The chart shows the percentage of β -gal expressing cells in each case. Numbers represent the mean values from three different transfections and the standard deviations are also shown. **B.** The levels of FLAG-PMLIV and VI at day 12 were then analysed by western blotting with an anti-FLAG antibody (M2). The GFP levels of a cotransfected GFP expression plasmid (Clontech) were also assessed to check for equal efficiency of transfection.

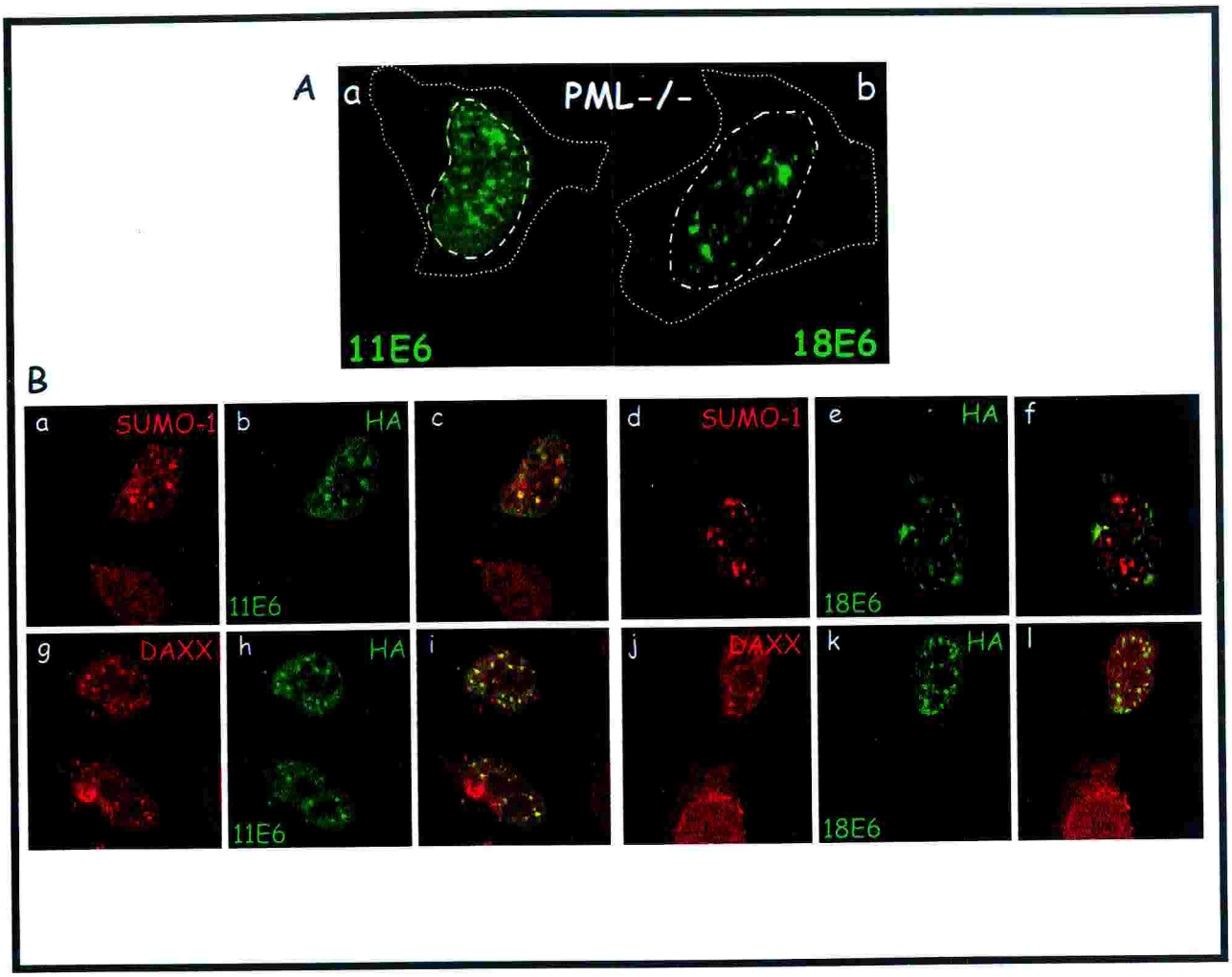

Fig. 30 Panel A. HA-E6 localisation in transiently transfected PML-/- cells. PML-/- fibroblasts were fixed in 3% PFA, permeabilized with Triton X-100 and stained for HA-11E6 (a), and HA-18E6 (b) using an anti HA rabbit polyclonal antibody (Y-11, Santa Cruz). **Panel B.** SUMO-1 localisation in PML-/- fibroblasts in the presence of HPV-11 E6 (a-c) or HPV-18 E6 (d-f). DAXX localisation in PML-/- fibroblasts in the presence of HPV-11 E6 (g-i) or HPV-18 E6 (j-l). PML-/- fibroblasts were fixed as in panel A and stained for HA-11 E6 (a-c) and (g-i) or for HA-18 E6 (d-f) and (j-l) using an anti HA rabbit polyclonal antibody (Y-11, Santa Cruz).

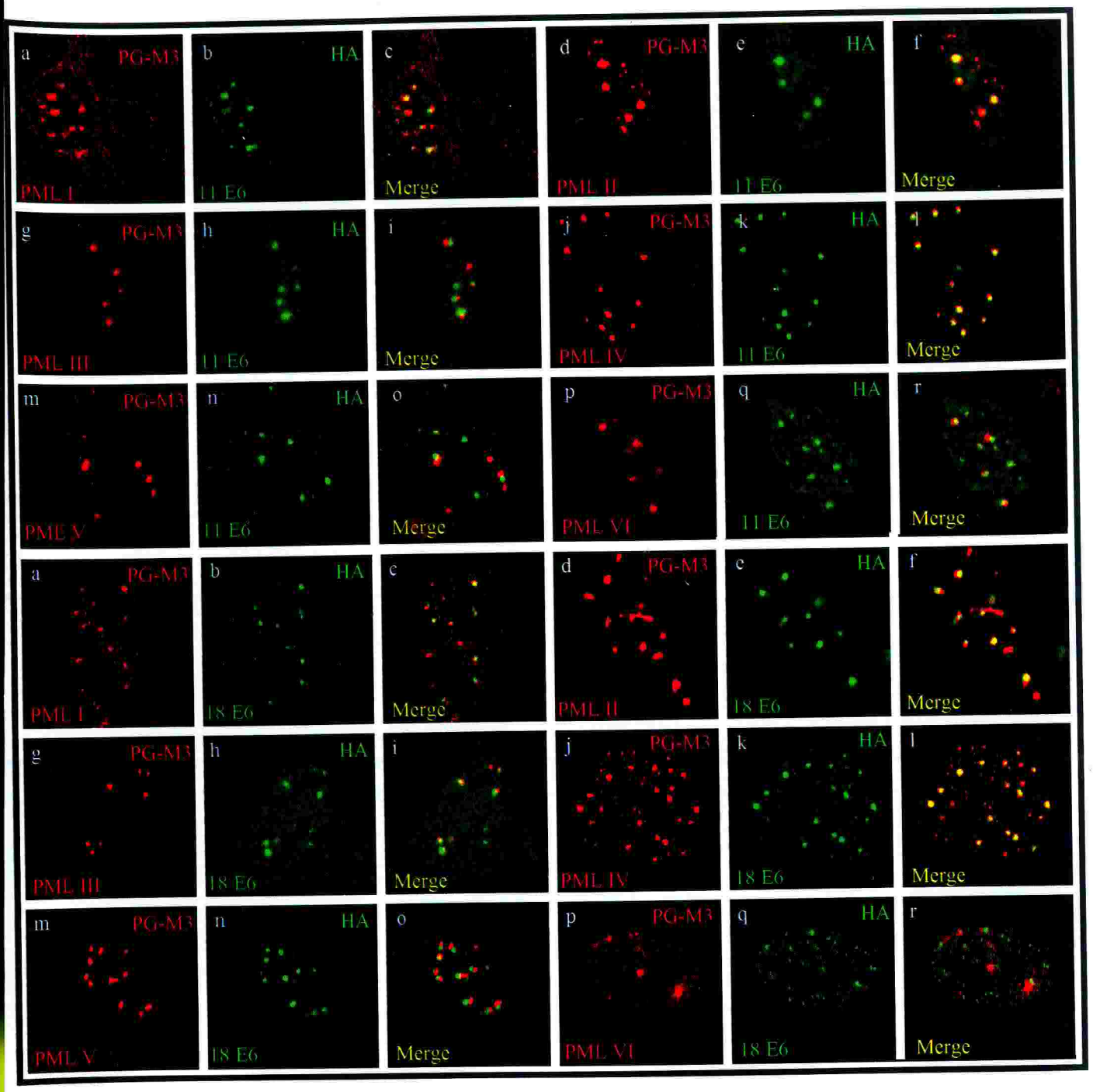

Fig. 31 HA-E6 colocalise with PML I, II and IV in transiently transfected PML-/- cells. PML-/- fibroblasts were fixed in 3% PFA, permeabilized with Triton X-100 and stained for HA-11E6 (a-l), and HA-18E6 (m-y) using an anti HA rabbit polyclonal antibody (Y-11, Santa Cruz) and anti FLAG mouse monoclonal antibody (M2, Sigma). The PML isoforms analysed are as follows: isoform I (a-c), isoform II (d-f), isoform III (g-i), isoform IV (j-l), isoform V (m-o) and isoform VI (p-r).

E6 localises to nuclear domains even in the absence of PML in PML-/- fibroblasts.

The analysis of protein distribution in PML-/- fibroblasts has revealed that proteins that are normally resident in PODs are dispersed throughout the nucleoplasm in the absence of PML (Lallemand-Breitenbach et al., 2001). As far as viral proteins are concerned, some of them are still present at sites of pre-existing PODs after their disruption and these sites coincide with viral replication centers (Ahn and Hayward, 1997; Ahn et al., 1999). In order to assess the localization of the E6 proteins in the absence of PML, HA-11 E6 and HA-18 E6 expressing plasmids were transfected into PML -/- fibroblasts (kindly provided by P.P. Pandolfi and G.Del Sal). Cells were fixed and stained after 24 hours as previously reported for the other cell lines used in this thesis. Surprisingly both E6 proteins still displayed a dot-like distribution even in the absence of PML (**Figure 30 A**). Even more interestingly, when these E6 transfected cells were double stained for proteins that normally reside within PODs, but which are normally diffused throughout the nucleus in the PML null cells, such as DAXX and SUMO-1, I also observed co-localization with E6 (see the yellow signal in the "merge" imaged, **Figure 30 B and C**). This suggests that E6 can act upstream of PML and might be crucial during differentiation and cell cycle progression in orchestrating PODs functions by recruiting PODs components such DAXX and SUMO-1 independently of PML.

E6 proteins are confocal with PML I, II and IV in a PML null background.

Since POD structures are likely to contain multiple PML isoforms I reintroduced both E6 and the different PML isoforms (I-VI) into $PML^{-/-}$ cells, to further define the nature of these

complex interactions that have so far been described. This experiment was therefore intended to determine which PML isoforms could co-localise with E6 independently of the other isoforms. $PML^{-/-}$ cells were not easily transfectable, thus although the experiment was repeated four times, I was not able to count more than 50 positive cells. Interestingly however , I observed that the only PML isoforms that were clearly confocal with the E6 proteins in the absence of the other isoforms were PML I, II and IV (**Figure 31**). No confocality could be detected between E6 and PML isoforms III, V and VI. Therefore these results suggest that PML I, II and IV are the isoform truly mediating the interaction with the E6 proteins.

E6 localises to nuclear domains enriched in proteasomes and poly-ubiquitinated proteins

So far I have shown that HPV-11 E6 and HPV-18 E6 mainly localise in cell nuclei where they accumulates in PODs. In an attempt to further characterize the composition of these E6-containing nuclear structures, I co-stained U2-OS cells for a series of cellular proteins by immunofluorescence analysis. The results show DAXX, SUMO-1 and PML to be mainly confocal with both E6 proteins, similarly to what I previously described in the case of PML-/- fibroblasts (**Figure 32 A**). Both DAXX and SUMO-1 are present in PODs as well as in the cytoplasm. The degree of confocality with the over-expressed E6 proteins is more evident in the case of SUMO-1, that is entirely accumulating in PODs, while DAXX retains its partial nucleosolic staining. Interestingly, I found the 20S proteasome subunit to be recruited to these structures (**Figure 32 B**), while proteasomes normally accumulate around the nuclear compartment and in a diffuse manner inside the nucleus. I also co-stained for poly-ubiquitinated proteins (FK2 anti poly-ubiquitin monoclonal antibody) and the co-localization was clear for both HPV-11 E6 and HPV-18 E6, suggesting that the E6

induced dots were active sites of proteasomal degradation (**Figure 32 B**). Having observed the concomitant presence of PML, E6 and the proteasome in these nuclear dots, I next wanted to assess whether PML could negatively regulate p53 degradation induced by E6, as recently reported for hdm2 (Louria-Hayon et al., 2003a). Saos-2 cells (p53 $^{-/-}$) were transfected with a PCDNA3-p53 expression plasmid, alone or in combination with PML IV, 18E6 or hdm2. The result shown in **Figure 32 C** clearly shows that PML cannot protect p53 from E6-induced degradation (compare lane 3, where PML IV is not expressed with lane 5, where PML IV is expressed), while it does so in the case of hdm2 (compare lane 6 where PMLIV is not expressed with lane 7, where PML IV is expressed). The effect of PML IV alone on the levels of p53 are marginal in this system (compare lane 2 with lane 4).

E6AP is recruited into PODs by E6

I next wanted to assess if PODs enriched in E6, proteasome components and polyubiquitinated proteins were also containing one of the ubiquitin ligases utilized by E6 to degrade some of its protein targets (Scheffner et al., 1990; Thomas and Banks, 1998; Nakagawa and Huibregtse, 2000). Previous reports have demonstrated the localization of murine mE6-AP both in the nucleus and in the cytoplasm (Hatakeyama et al., 1997). In this study I made use of human E6AP with a Green Fluorescent Protein (GFP) tag; its localization is mainly cytoplasmic with faint nuclear staining, in accordance with previous data (**Figure 33 a-c**). Co-expression of HPV-18 E6 leads to a clear relocalization of E6AP to the nucleus and its accumulation in discrete dots (**Figure 33 d-f**). At the same time the two proteins also display a high level of confocality in the cytoplasm. This interaction is highly specific, since both HPV-11 E7 and HPV-16 E7 localise to similar dots in the

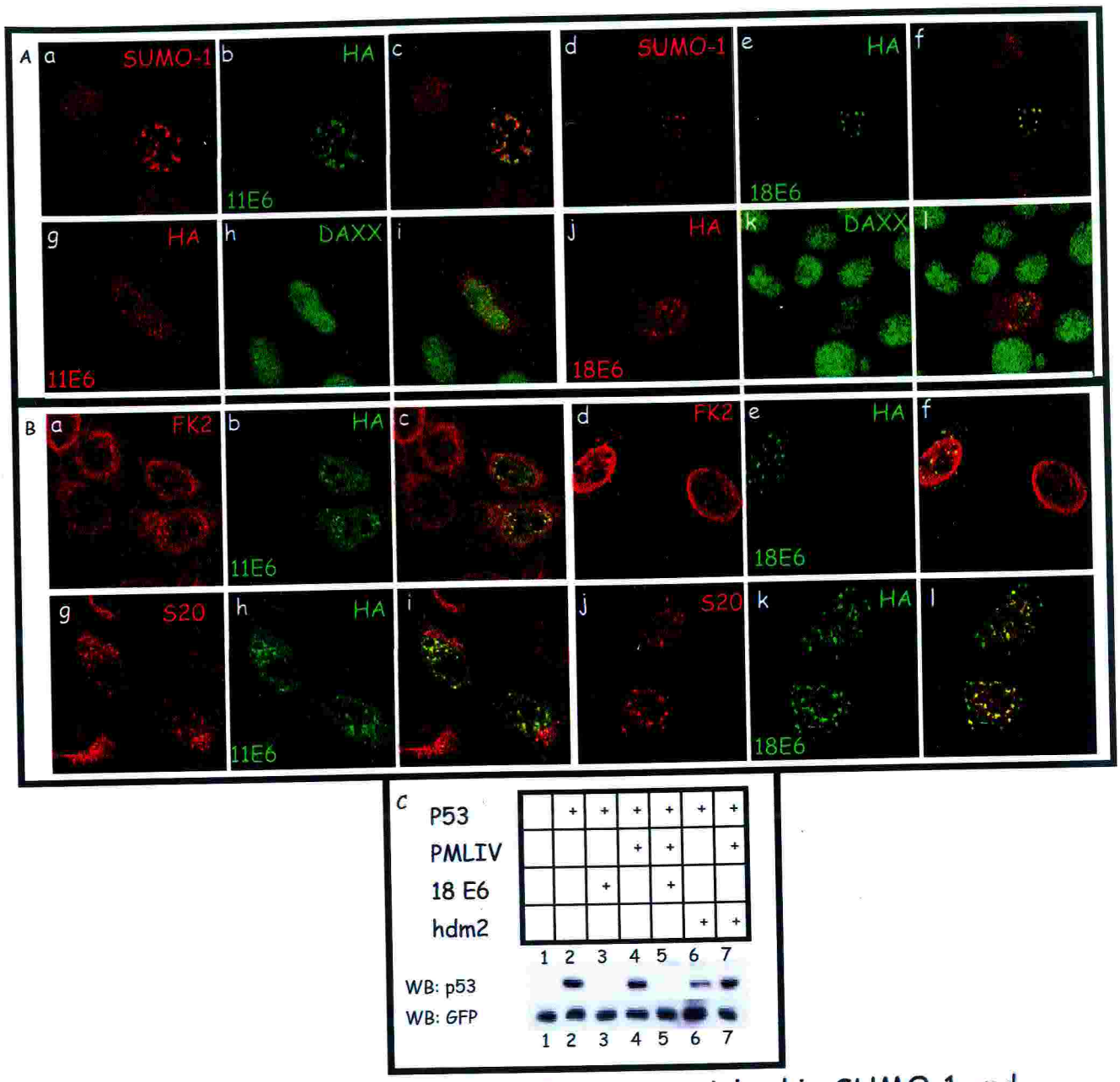

Fig. 32 Panel A. E6 containing PODs are enriched in SUMO-1 and DAXX proteins. U2-OS cells were transiently transfected with HA-11 E6 (a-c and g-i) and HA-18 E6 (d-f and j-l), fixed as described previously and stained with anti-SUMO-1 (a-f) and anti-DAXX (g-l) antibodies. **Panel B.** E6 containing PODs are enriched in poly-ubiquitinated proteins and proteasomes. U2-OS cells were fixed and stained with anti-poly ubiquitin (a-f) and anti-proteasome (g-l) monoclonal antibodies (Affinity) and double stained for the presence of HA tagged E6. **Panel C.** PML does not rescue p53 from E6 mediated degradation. Western blot analysis of transiently transfected Saos-2 cells (p53 -/-). PCDNA3-p53 was transfected alone or in combination with the indicated plasmids. Lane 5 shows no rescue by PML of E6 mediated degradation of p53. Lanes 6 and 7 show the opposite in the case of hdm2.

nucleus (**Figure 9 and Figure 24**), but fail to recruit E6AP to these structures (**Figure 34**). Having demonstrated that E6 is confocal with PML IV in nuclear domains, we tested whether PML IV over expression could also recruit E6AP independently of E6. U2OS cells were transfected with the GFP-E6AP expression construct, together with FLAG-PMLIV alone or in combination with HA-18E6. The result in **Figure 33 g-i** shows that PML is not sufficient for E6AP recruitment to PODs, while in the presence of E6 the three proteins (E6-E6AP-PMLIV) clearly colocalise (**Figure 33 j-l**). The latter cannot be directly visualised due to the unavailability of a triple staining system, however by comparing panel g-i with panels j-l, it is clear that E6 is the driving force that targets E6AP to PODs.

GFP-E6AP colocalises in nuclear structures with E6 and PML isoforms I-IV, but not with PML isoforms V and VI.

I next wanted to verify whether or not E6AP was being specifically recruited by E6 to the same PODs structures within the nucleus. Having observed that PML by itself is not sufficient to recruit E6AP (Figure 33 j-l), I proceeded to transfect into U2-OS cells, constructs expressing both GFP-E6AP and the different PML isoforms (I-VI), in the presence of the HA-18 E6 construct. The latter could not be visualised due to the unavailability of a triple staining system. What is clear however from **Figure 35** is that E6AP localizes similarly to HA-18E6 in respect to the different PML isoforms, being confocal with PML I-IV (**Figure 35 a-l**), but not with PML V and VI (**Figure 35 m-r**). These results thus further demonstrate that E6 can target E6AP to specific PODs structures, which are also enriched in components of the proteasome machinery and thereby suggest that this is where E6 mediated degradation of some of its substrates takes place.

E6 can enter the nucleus together with E6AP, subsequently targeting PODs.

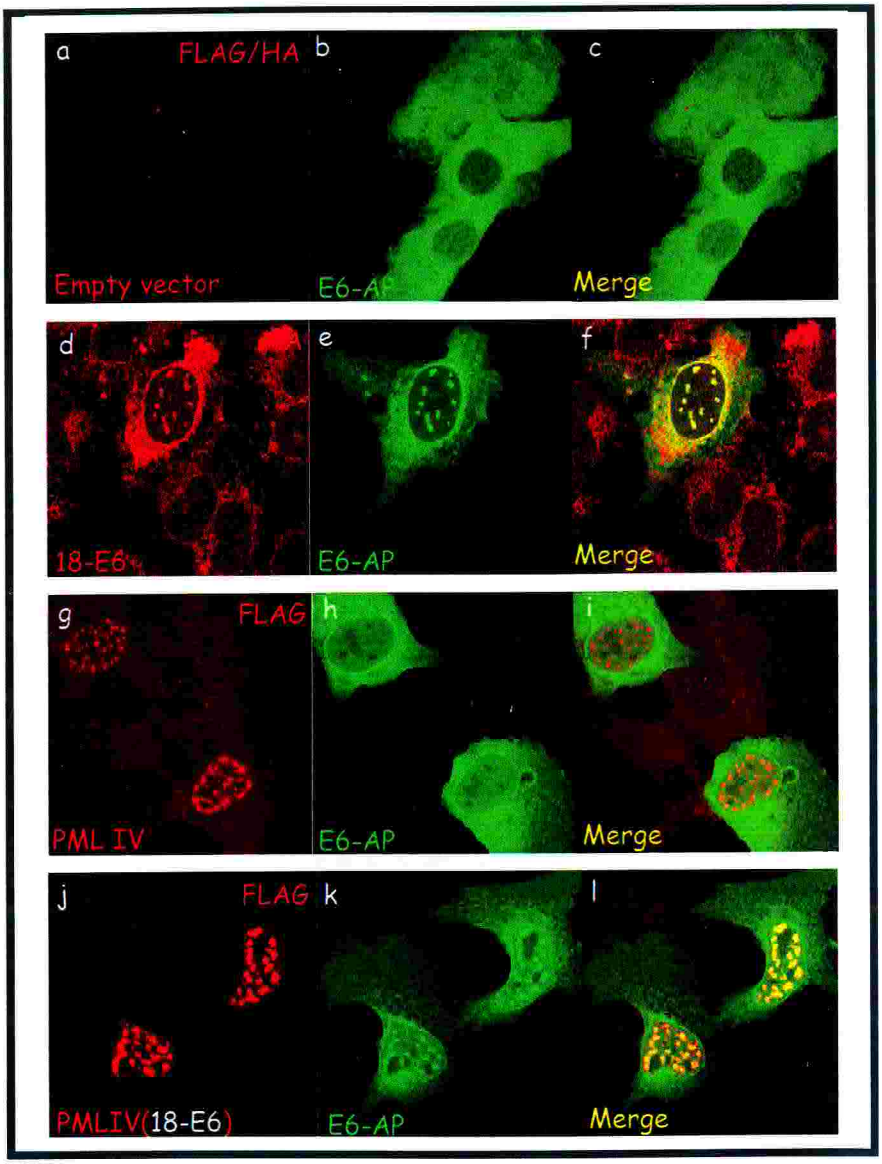

Fig. 33 GFP-E6AP colocalises in nuclear structures with E6 and PML. U2-OS cells were transiently transfected, fixed and stained for HA-E6 (Y-11, Santa Cruz) or Flag-PML IV (M2, Sigma). Panels a-c show E6AP alone, panels d-f E6AP together with 18E6, panels g-i E6AP in combination with PML IV, and panels j-l show the three proteins expressed together, although cotransfected E6 is not detectable in this case, due to the impossibility of performing a triple staining.

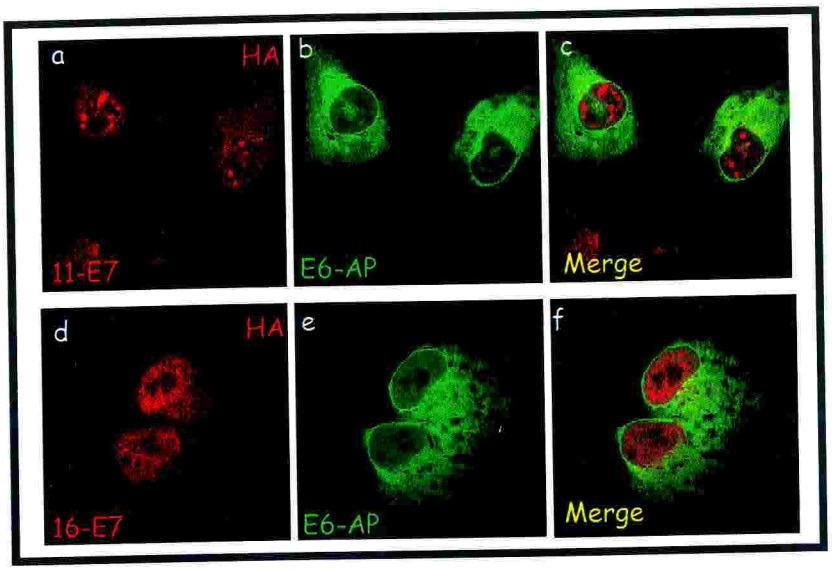

Fig. 34 GFP-E6AP does not colocalise with 11E7-induced structures. GFP-E6AP was transiently transfected together with HA-11 E7 or HA-16 E7. Cells were fixed and stained with anti HA (Y-11, Santa Cruz). GFP-E6AP together with HA-11 E7 (a-c), and GFP-E6AP together with HA-16 E7 (d-f)

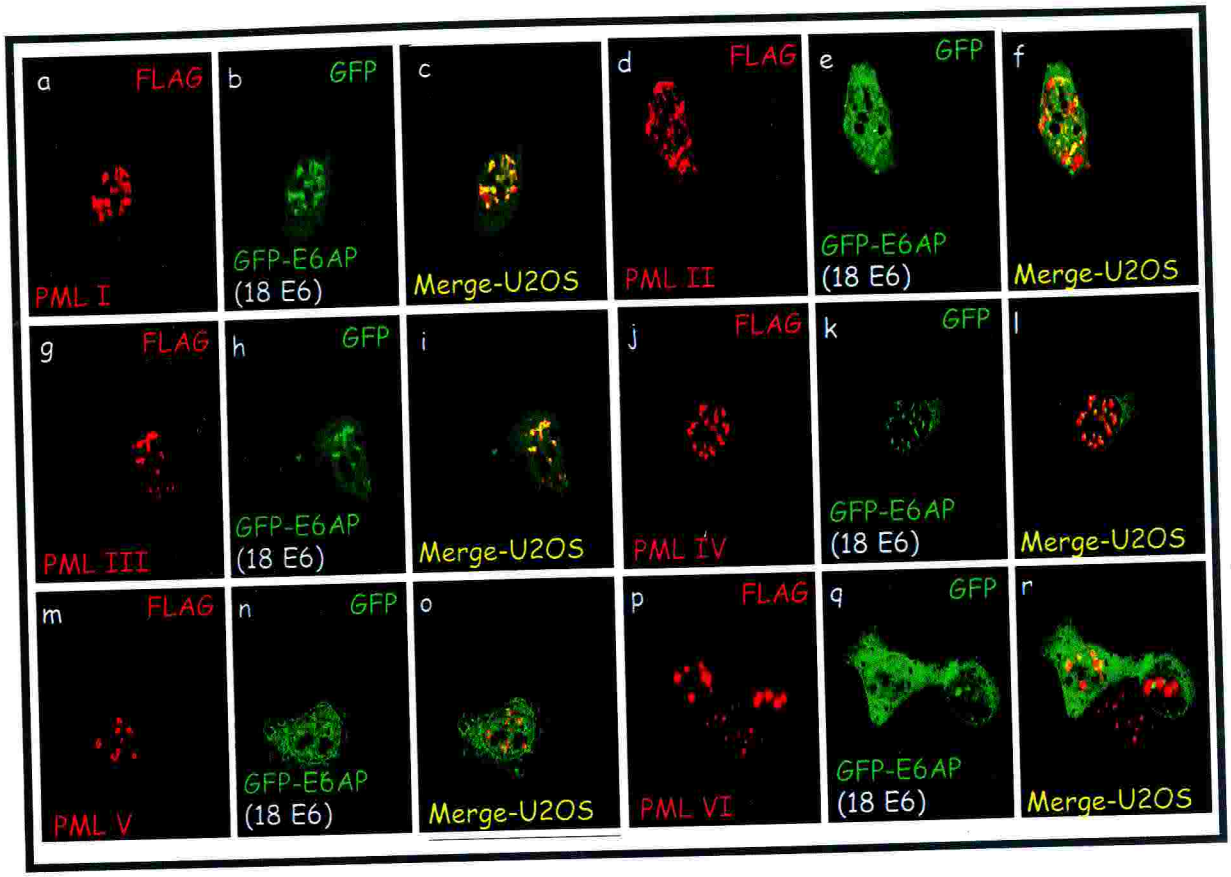

Fig. 35 GFP-E6AP colocalises in nuclear structures with E6 and PML isoforms I-IV, but not with PML isoforms V and VI. U2-OS cells were transiently transfected with GFPE6AP, HA-18E6 and PML expression plasmids, fixed and and stained for Flag-PML IV (M2, Sigma), in the presence of HA-18 E6. Although cotransfected E6 is not detectable, due to the impossibility of performing a triple staining. Panels a-c show E6AP in combination with PMLI, d-f with PMLII, g-i with PMLIII, j-l with PMLIV, m-o with PMLV, and p-r with PMLVI.

HPV-16 E6 has been recently shown to enter the nucleus through a number of different mechanisms, all of which involve a Nuclear Localization Signal (NLS) present in the C-terminal portion of the protein (Le Roux and Moroianu, 2003). I next wanted to determine whether the NLS present on HPV-18 E6 was required for getting E6-E6AP into the nucleus and I made use of the NLS mutants described in Figure 10-11. As predicted the resulting E6 proteins, when expressed in cells, localized in the cytoplasm (**Figure 36 a-b** and Fig.11A 2-3) and a diffuse cytoplasmic localization was observed in the case of GFP-E6AP alone (**Figure 36 c**). Interestingly when I co-expressed E6AP together with the 18 E6 NLS1 or the 18E6 NLS2 mutant I saw the recruitment of both proteins to nuclear structures (**Figure 36 d-f and g-i**). This indicates that E6 can enter the nucleus by alternative ways, not all of them necessarily depending on its C-terminal NLS, and that E6 AP is a protein that may be able to mediate this "piggy back" entry of the E6 proteins.

P53 is recruited and degraded in E6 induced nuclear domains.

The loss of p53, promoted by the E6/E6AP complex, is considered to be an essential step in the initiation of cervical cancer. Previous studies have reported the localization of endogenous p53 and E6 in different cell lines to be mainly cytoplasmic, but interestingly p53 also had a weak punctuate staining in the nuclei of HPV positive HeLa and CaSKi cells. This nuclear staining was absent in HPV negative cells (Liang et al., 1993). To further analyse this, I performed immunofluorescence analysis on Saos-2 p53 null cells. Over-expression of p53 in Saos-2 cells leads to a clear detection of the protein in the cell nucleus (**Figure 37 a-c**). Co-expression of HPV-18 E6 leads to p53 degradation, but interestingly p53 was still detectable in E6-induced nuclear dots (**Figure 37 d-f**). In accordance with previously published literature, E6AP is not able to degrade p53 in the

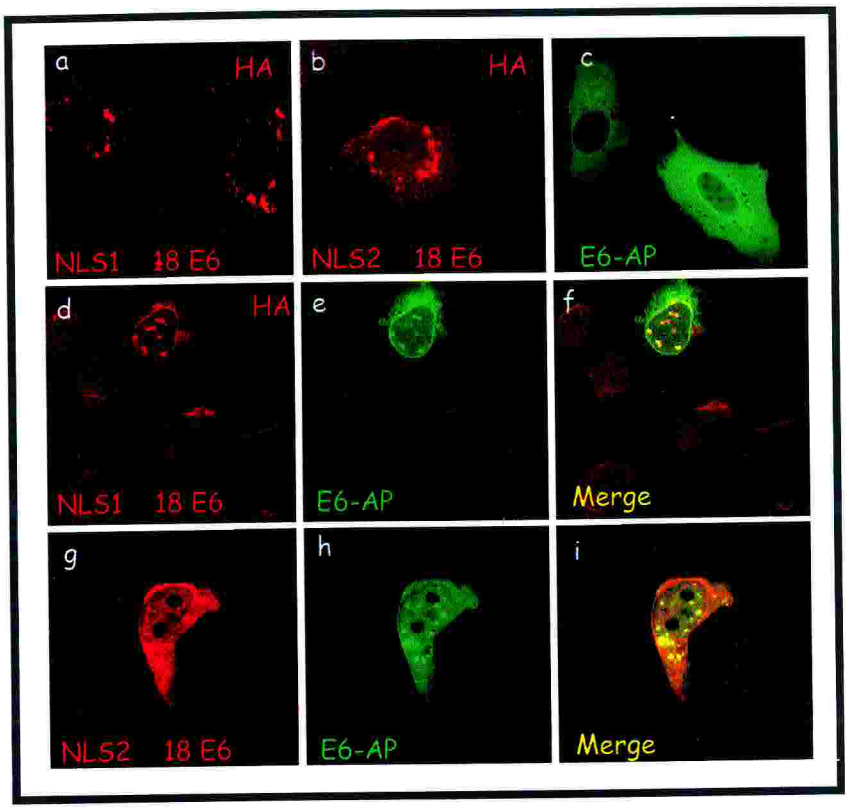

Fig. 36 E6 mutants, mutated in its NLS (NLS1 and NLS2) are still able to enter the nucleus and to form nuclear structures together with E6AP. U2-OS cell were transfected with GFP-E6AP alone or together with HA-18 E6 NLS1 (RRR 124-126 AAA) or NLS2 (RR153-154GG). a: HA-18 E6 NLS1 alone, b: HA-18 E6 NLS2 alone, c: GFP-E6AP alone, d-f: GFP-E6AP and HA-18 E6 NLS1 together, g-i: GFP-E6AP and HA-18 E6 NLS2 together.

absence of E6 and retains a clear cytoplasmic localization (**Figure 37 g-i**), however in the presence of E6, E6AP is then recruited to nuclear dots, where p53 is readily degraded (**Figure 37 j-l**).

These results raised the possibility that PODs were the site of E6-induced degradation of p53. If this was indeed the case, then I should expect to detect p53 accumulation at these sites, shortly after proteasome inhibition. In order to investigate this possibility, I used a HPV positive cell line, CaSKi, and as a negative control a HPV negative cell line, HaCaT. The results obtained are shown in **Figure 38**. HPV positive CaSKi cells were treated for 15 (**m-o**) and 20 minutes (**p-r**) with N-CBZ and with DMSO as a control (**j-l**). HPV negative HaCaT cells were also used for comparison (**a-i**). The co-localization observed between endogenous p53 and PML is not complete, but is readily detectable after 15 minutes which is visible in the enlarged image. This result further confirms the idea of specific PODs, being targeted by HPV E6 as sites of active proteasomal degradation. This degradation is induced, in the case of p53, by the concomitant presence in PODs of the ubiquitin ligase E6AP. At present no other ligases have been specifically implicated in E6-mediated ubiquitin conjugation, however it will be interesting in the future to investigate whether or not these other ligases are present in PODs, together with E6.

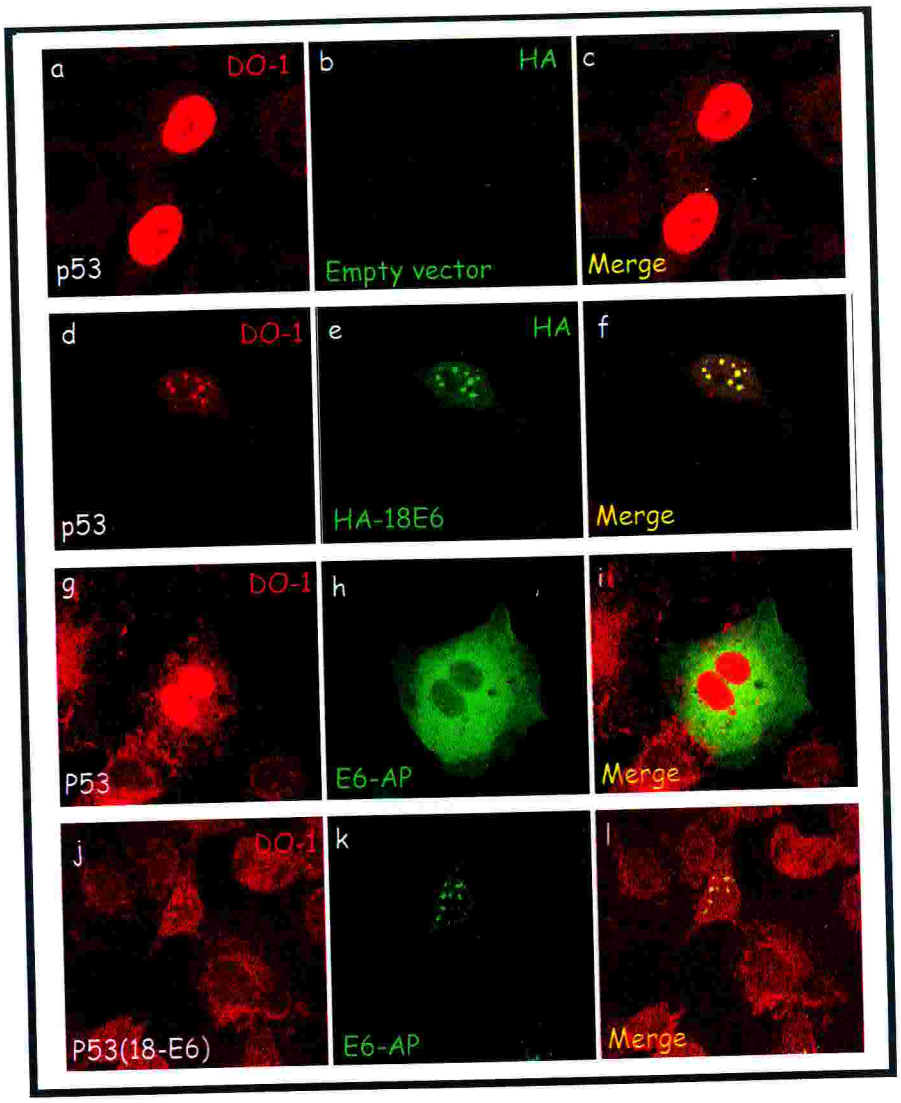

Fig. 37 E6 containing nuclear bodies also contain p53. SaOS-2 cells were transfected with a p53 expression construct alone (a-c) or with HA-18 E6 (d-f), with GFP-E6AP (g-i) or in combination with HA-18 E6 and GFP-E6AP (j-l), although cotransfected E6 is not detectable in this case, due to the impossibility to perform a triple staining. Cells were stained with a p53 monoclonal antibody (DO-1, Santa Cruz), and with an anti-HA rabbit polyclonal antibody (Y-11, SantaCruz).

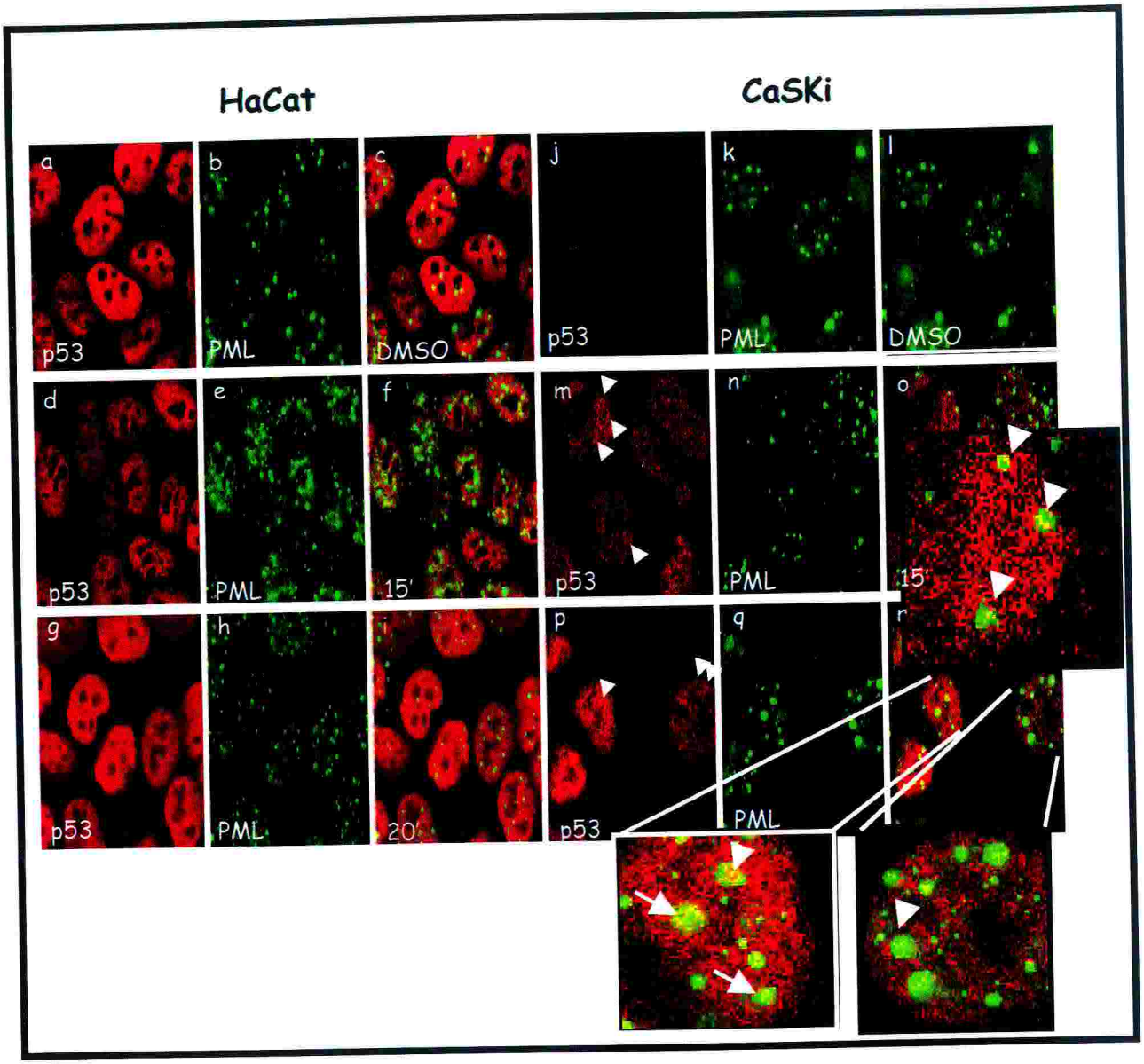

Fig. 38 Endogenous p53 accumulates in PODs after 15-20 minutes of proteasome inhibition treatment. HPV negative HaCaT cells and HPV positive CaSKi cells were treated for the indicated time with N-CBZ and then fixed and stained for p53 (DO-1, Santa Cruz), and for PML (H-238, Santa Cruz). Blown-up images are shown for the most relevant cells.

DISCUSSION

The post-genomic era has changed our concept of proteins as acting separately within the cell, so that we now have to view them as part of complex networks of dynamic and highly regulated interactions, that control cell physiology, and development of the organisms to which they belong. In this context, it is essential to consider this complex regulation at all levels, going from transcriptional to translational regulation, from modulation of protein activity by post-translational modifications to regulation of the sub-cellular localization of single proteins and of macromolecular complexes. In this thesis I have elected to investigate this last point in detail, concentrating my attention on two viral oncoproteins that are essential for the HPV life cycle and for its tumourigenic potential: E6 and E7.

These two proteins have been shown to interact with a variety of cellular targets (Mantovani and Banks, 2001; Munger et al., 2001), and to be required for the normal productive viral life cycle as well as for the malignant transformation brought about by HPV (Song et al., 1999; Flores et al., 2000). Recent studies have sought to investigate their function by targeting their expression to the epidermis or the developing ocular lens in transgenic mice. The results obtained are tissue dependent, and there is some variability among the different studies (Song et al., 1999; Riley et al., 2003). Nonetheless the general model that can be derived is that E7 mainly contributes to the early stages of tumour promotion, inducing the formation of benign tumours, while E6 acts primarily as a "progression factor", accelerating progression of these benign tumours to the malignant stage. Consistent with this model, E6 and E7, when expressed together, produce larger and more invasive malignancies in double transgenic mice (Song et al., 2000). A question that has remained elusive, however, is how relatively low abundant proteins such as E6 and E7

can interact with so many cellular partners in a coordinated and regulated way, and the data presented in this thesis may help to answer this question, by showing that both proteins localise to PML Nuclear Bodies.

In order to investigate further the complex interactions between the E6 and E7 proteins and the cellular environment, I initially compared the localization of two E7 proteins, one derived from a mucosal low-risk virus, HPV-11, and the second one from the mucosal high-risk HPV-16. The first interesting observation is that the low-risk HPV-11 E7 protein has a tendency to accumulate in dot-like structures within the nucleus, and I have identified these as being PML Oncogenic Domains also known as PODs or ND10. In contrast the high risk HPV-16 E7 protein, while also localising predominantly to the nucleus, does not mainly accumulate in PODs. This first finding was then further confirmed by staining for a second PODs component, SUMO-1. SUMO-1 is a small ubiquitin-like protein, which has been shown to bind covalently to, and modify specific nuclear forms of, Sp100 and PML (Boddy et al., 1996; Sternsdorf et al., 1997b), and these modified forms are the ones responsible for correct PODs assembly (Ishov et al., 1999). The observed difference between the localization of low- and high-risk HPV-E7 could simply be due to the fact that the high-risk protein is not present in PODs, or alternatively, is just not predominantly present at these sites. When HPV-16 E7 and PML IV are co-expressed in a transient transfection assay, the two colocalise in PODs, and this result would be in favour of the latter hypothesis. A different PODs "in-out rate" of the two viral proteins could easily explain this, with HPV-16 E7 possibly being more tightly regulated at the post-translational level than HPV-11 E7. The latter explanation is in my opinion the most probable, since E7 has been shown to interact with several proteins that are normally resident in PODs, such as pRb (Munger et al., 1989b), HDAC1 (Brehm et al., 1999), the Ski/Skip complex (Prathapam et al., 2001) and CBP/p300 (Bernat et al., 2003), thus making it highly

improbable that HPV-16 E7 shows complete exclusion from these domains. Consistent with this, it is also known that proteins that are present in PODs, such as CBP and pRb, are in turn highly regulated, with a high turnover both in and out of PODs (Alcalay et al., 1998; LaMorte et al., 1998). Unlike the situation with the Adenovirus E1A protein however, an E7 mutant that lacks the pocket protein interaction domain (E7 p24Gly) is still able to accumulate in dots. This observation excludes pRb as the possible mediator of E7-PML interaction and could be possibly explained by the interaction between E7 and CBP/p300, being only partially LXCXE dependent, or by the interactions with HDAC1 and Skip that rely on amino acid residues which reside outside the CD2 portion of the E7 protein (Brehm et al., 1999; Prathapam et al., 2001).

What is clear however is that the presence of E7 in PODs has the potential to have multiple outcomes. First of all, PODs have been shown to accumulate components of the proteasome machinery (this thesis and (Lallemand-Breitenbach et al., 2001)), thus PODs could be the site where pRb is directly inactivated and its degradation induced by E7. Unfortunately the E3 ubiquitin-ligase utilized by E7 still remains to be identified, but once identified, it will be interesting to assess its localization in HPV positive and negative cell lines. Moreover PODs are also enriched in the USP7/HAUSP ubiquitin protease (Everett et al., 1997), and it would be interesting to see, given the localization in the same nuclear domains, if it is also involved in regulating the stability of E7 specific targets. This same hypothesis is also valid in the case of the E6 proteins that, as demonstrated by the data presented in this thesis, also have the tendency to colocalise with PML within the nucleus. Another interesting point is that PML positively regulates presentation of MHC class I antigens, enhancing the expression of key regulatory proteins (Zheng et al., 1998), thus it is possible that either E7, or E6, might alter such important antiviral regulation. In support of this, the E7 oncoprotein has been shown to repress the major histocompatibility complex

(MHC) class I heavy chain promoter, as well as the promoter of the transporter associated with antigen processing subunit 1 (TAP1) promoter (Georgopoulos et al., 2000).

A fact that is important to note is that the low-risk HPV-11 E7 preferentially accumulates in these domains, and non oncogenic E7 proteins have also been shown to bind pRb, without destabilising it, thereby activating E2F dependent promoters (Ciccolini et al., 1994). This association seems to be much less dynamic then that of the high-risk HPV-16 E7. Whether this is related to differences in how the two proteins interact with the proteasome machinery remains to be determined. Thus the presence of the E7 protein in PODs could be related to the de-repression of E2F promoters, independently of pRb degradation, and could in turn be mediated by the interactions with transcriptional co-activators and co-repressors such as p300 and HDAC1 (Brehm et al., 1999; Bernat et al., 2003).

One of the questions that I addressed next, was how E6 nuclear entry occurs and how this is regulated. There are three major points that arise from my results that I think are important. Firstly HPV-18 E6 has two putative Nuclear Localization Signals (NLS), at positions 124-126 and 153-154, that when singularly mutated strongly inhibit E6 nuclear entry. This is consistent with what has been recently demonstrated for HPV-16 E6 (Le Roux and Moroianu, 2003), and confirms that this is a functional bipartite NLS (Fontes et al., 2000). The second observation is that, although reduced for nuclear entry, these NLS mutants are still able to enter the nucleus, suggesting that there are alternative ways by which this can occur. Passive diffusion is one possibility, given the small size of the E6 protein (Rout and Aitchison, 2001; Vasu and Forbes, 2001; Pante and Kann, 2002), while the entry by "piggy-back" on a second protein, that is in turn actively transported, is also a possibility. This latter point was further investigated, and the results indicate.that E6AP is one of the possible proteins mediating this "piggy-back" entry. This is the third interesting

point, since the E6-E6AP complex is preferentially targeted to nuclear PODs, and E6AP is able to mediate nuclear entry even in the absence of a functional NLS on E6. This last point has important implications for the degradation of E6AP's normal substrates as opposed to the ones to which E6AP is re-directed by HPV E6. E6AP can indeed bind and degrade several substrates in the absence of E6, such as some members of the Src family tyrosine kinases (Oda et al., 1999), HHR23A, which is one of the human homologues of the yeast DNA repair protein Rad23 and is involved in DNA repair (Kumar et al., 1999). Others include Bak, a proapoptotic member of the Bcl-2 family (Thomas and Banks, 1998) and MCM7 (multicopy maintenance protein 7), which is a subunit of the replication licensing factor-M, involved in replication (Kuhne and Banks, 1998). The relocalization of E6AP to PODS mediated by E6 can direct the ubiquitin ligase to other cellular targets, mainly present in the nucleus, which are not normally targeted by E6AP. P53 is a perfect example of this.

Next I proceded to analyse the mechanism of action of the E6*I protein upon the functions of the full length E6 protein, beginning with an investigation of the respective localizations of these two different proteins within the cell. I first showed that a construct comprising the cDNA of HPV-18 E6 also expresses HPV-18 E6*I, whose splice donor and acceptor sites both lie within the full-length HPV-18 E6. To produce a construct that expresses only full length HPV-18 E6, I mutated the splice donor site, generating constructs expressing a non splicing full length HPV-18 E6. I also made use of a construct expressing E6*I alone. This Non Splicing mutant (HPV-18E6 NS) was tested for its functionality and still retained the ability to degrade p53. Using HA tagged versions of the two proteins I then analysed their pattern of expression. Both the full length and E6*I localize throughout the cell, with full length E6 preferentially accumulating in discrete dots in the nucleus that I have demonstrated to be confocal with PML.

Since I had the possibility to monitor the expression of the E6*I protein in cells, I also investigated the effects of a forced reintroduction of this alternatively spliced form on E6-induced degradation of its cellular targets in HPV- containing cells. I reasoned that this could simulate a situation in which higher levels of E6*I are produced inside the cell, as transcripts expressing E6* mRNAs are the most abundant in the lower levels of the infected epithelia (Bohm et al., 1993; Nilsson et al., 1996). From this experiment I could detect a clear increase in the levels of one of the main targets of the E6 protein: the p53 protein (Scheffner et al., 1990), no increase was observed for two other known targets of E6, hDlg and Scribble. This result could have three explanations. The first is that this interaction results in the rescue of those targets in which E6AP is the ubiquitin ligase involved, but not those targets in which other ligases may be involved, and this is in accordance with our previous data suggesting the existence of alternative ligases used by E6 (Pim et al., 2000). A second explanation could have to do with differences in the respective localization of these E6 targets within the cell. In this respect E6*I might be able to rescue only the nuclear targets of full length E6, and not the others. A third, and more trivial explanation, could relate to the low efficacy of this rescue and to the sensitivity of the immunofluorescence detection. This result provides a molecular explanation for previous data from this lab, showing that E6*I overexpression was sufficient to induce apoptosis in HPV positive cells (Pim and Banks, 1999). Interestingly, I also found that the levels of E6*I expression vary during the cell cycle with high levels present in G2/M, while the levels of the full length E6 protein remain constant.

The major alterations in the cell cycle machinery which accompany cervical carcinogenesis, have been shown to be reversible upon over expression of the E2 protein, which normally regulates the levels of E6 and E7 expression (Goodwin and DiMaio, 2000). In the model that I can propose the high-risk viruses have E6*I, and most likely E6*II-IV,

as an additional level of control in addition to that of E2. The E6*I protein seems to modulate E6 by inhibiting at least some of its functions, thereby allowing the virus to more tightly control the initial stages of the infection, where following entry into basal epithelial cells, HPV genomes are maintained as autonomous replicating elements and a low level of HPV protein expression occurs. A correct regulation of mitosis is essential at this stage, and this is where p53 levels might need to be increased (Stubenrauch and Laimins, 1999). This is consistent with the observation that p53 is not completely degraded in HPV positive cells (Cooper et al., 1993; Mantovani and Banks, 1999), and although p53 has been shown to specifically inhibit HPV replication in vivo (Lepik et al., 1998), its proofreading activity might be required, at low level, to ensure the correct replication of the HPV genome. P53 has indeed been implicated in direct stimulation of topoisomerase I activity, and wild-type p53 allows a more efficient recruitment of topo I after DNA damage (Mao et al., 2000). Moreover p53 possesses 3'-5' exonuclease activity, with high efficacy in removing terminal mismatches, and such activity has been proven important to increase the fidelity of DNA synthesis by Human Immunodeficiency Virus-1 Reverse Transcriptase (HIV-1 RT) (Bakhanashvili, 2001b), as well as by murine leukemia virus Reverse Transcriptase (MLV-RT) (Bakhanashvili, 2001a). Similar functions may be required for HPV as well. P53 has indeed been shown to interact with the E2 viral replication factor (Massimi et al., 1999) and might be present in viral replication centers, similarly to what has been already demonstrated in the case of CMV (Fortunato and Spector, 1998) , HSV (Wilcock and Lane, 1991), and Adenovirus (Konig et al., 1999). The results presented in this thesis are in support of this hypothesis, showing that p53 colocalises with E6 in PODs, where also E2, E1 and a viral ORI-containing plasmid have been already shown to localise (Swindle et al., 1999).

Over recent years PML has been the subject of intense investigation and it is now clear that PODs play a central role in regulating functions involved in cell cycle control, apoptosis and cellular senescence (Alcalay et al., 1998; LaMorte et al., 1998) (Fogal et al., 2000; Torr et al., 1999) (Ferbeyre et al., 2000; Pearson et al., 2000). Thus it was extremely interesting to find that the E6 proteins preferentially target these domains within the cell. An initial investigation suggested that the E6 proteins did not seem to disrupt PODs in the same way as reported for CMV, HSV-1, EBV and the Adenovirus immediate-early and early proteins (Everett and Maul, 1994; Ahn and Hayward, 1997; Ahn et al., 1998; Everett et al., 1998a; Muller and Dejean, 1999). The data presented in this thesis are instead similar to what has been observed in the case of SV40, where the Large T protein associates with p53, and the two proteins co-localise in dot like structures juxtaposed to PODs. Interestingly, this association and localization correlate well with the transformed phenotype induced by SV40 (Jiang et al., 1996). What is also interesting is that a similar localization is observed for both high- and low-risk E6 proteins, suggesting a common POD targeting mechanism.

Increasing evidence points at alternatively spliced PML isoforms as having different roles in diverse biological processes (Jensen et al., 2001; Bischof et al., 2002). Indeed recent studies have provided evidence for different PODs within the cell, with a dynamic composition, suggesting, possibly, different functions (Muratani et al., 2002; Wiesmeijer et al., 2002). In agreement with this concept, I have reported that the HPV-11 and HPV-18 E6 proteins are targeted to defined POD structures within the nucleus, which consist specifically of PML isoforms I-IV, but not PML isoforms V and VI. These results demonstrate that POD structures have different PML compositions and suggest that the viral E6 proteins target only a subset of those domains that are important for specific viral activities. One of the possible explanations for the presence of E6 in PODs was that E6 was being targeted to PODs via PML binding. In order to verify this hypothesis I proceeded to

investigate whether the HPV E6 proteins could interact with the different PML isoforms both *in vivo* and *in vitro*. The results obtained are consistent in both systems, pointing to PML isoforms I, II and IV as being the main interaction partners of the E6 proteins. Specifically, HPV-11 E6 shows a very strong interaction with PML isoforms I, II and IV both *in vivo* and *in vitro* and this is consistent with the *in vivo* co-localization data from the immunofluorescence analysis. I also consistently observe that PML isoforms V and VI, which fail to co-localise with 11 E6 in immunofluorescence experiments, also fail to interact either *in vivo* or *in vitro*. The results with isoform III are somewhat more difficult to interpret. Isoform III shows strong co-localization with 11E6, but does not appear to interact in the co-immunoprecitation assays. The fact that this isoform does not interact *in vitro*, however, suggests that the discrepancies observed may be due to the presence, in the same dots, of overexpressed isoform III as well as the other endogeneous isoforms, with which 11 E6 is truly interacting. Obviously I cannot conclude at this stage which (if any) of these interactions are direct and the contribution of other E6 target proteins within these different domains may also affect the pattern of E6 interactions. Nonetheless these studies provide very strong evidence that a subset of the PML protein, defined by isoforms I, II and IV are targets of the HPV-11 E6 protein.

The failure of HPV-18 E6 to be detected in the *in vivo* co-immunoprecipitation experiments with the different PML isoforms was somewhat surprising, considering its apparently equal propensity to co-localise with a subset of PML isoform containing POD structures and to bind them as efficiently as HPV-11 E6 *in vitro*. However, since HPV-18 E6 readily targets many of its substrate proteins for degradation, I reasoned that this might be an explanation for this discrepancy, and indeed, this would seem to be the case. Co-expression of HPV-18 E6 with PML isoform IV induces a dramatic reduction in the steady state levels of the insoluble portion of the protein, which can be rescued by treating the cells with a

proteasome inhibitor. Interestingly, PML VI, with which HPV-18 E6 does not co-localise either *in vivo* or *in vitro*, was also unaffected by the presence of the HPV-18 E6 protein. Therefore these studies suggest that HPV-18 E6 specifically triggers the proteolytic degradation of a portion of PML IV. Obviously it would be of great interest to discover whether this form of PML has different activities compared with the soluble form of the protein, which is not targeted by E6.

The accumulation of both high- and low-risk E6 proteins within PODs is intriguing. POD targeting by viral proteins has been found to be essential for an efficient viral life cycle in the cases of Adenovirus, EBV, HSV-1 and CMV (Everett and Maul, 1994; Ahn and Hayward, 1997; Ahn et al., 1998; Everett et al., 1998a; Muller and Dejean, 1999). In all these cases the viruses encode at least one protein that is able to target and reorganize PODs. In this thesis I have shown that E6 and E7 from both a high-risk and a low-risk virus localise to PODs, as do the E1 and E2 proteins from HPV-11 (Swindle et al., 1999). Moreover the L2 minor capsid proteins from both HPV and BPV co-localize with PML bodies and are able to recruit both the L1 major capsid protein and the E2 into PODs (Day et al., 1998; Heino et al., 2000). These studies point to PML Nuclear Domains as being central players at the later stages of viral replication and capsid assembly. This hypothesis is further supported by recent studies demonstrating that the L2 protein is also capable of inducing POD re-organization in differentiating epithelia (Florin et al., 2002a; Florin et al., 2002b).

In the case of the HPV E6 proteins the interaction with certain PML isoforms may have more profound implications for the subversion of the cell homeostasis. Recent studies have shown that PML isoform IV is a potent inducer of cellular senescence (Pearson and Pelicci, 2001; Bischof et al., 2002). Intriguingly, recent reports also showed that reducing HPV gene expression in cervical tumour derived cell lines could also result in an induction of

senescence (Wells et al., 2000). I reasoned that these two observations may be connected, and in agreement with this hypothesis, I have shown that both the HPV-11 and the HPV-18 E6 proteins are potent inhibitors of PML IV-induced senescence in primary epithelial cells. Hence, whilst inhibiting this function of PML may be an important element for the replication of both viruses, it may also represent a key step in the process of HPV-18 E6 induced malignant transformation. Another important point that merits discussion is the fact that the block of PML induced senescence seems to be largely p53 independent, being equally obtained by HPV-11 E6 and a mutant of HPV-18 E6 that is reduced in the ability to degrade p53. It is possible that the block of senescence by E6 may be mediated by its interaction with CBP/p300, that is also recruited to PODs upon the induction of senescence, and mediates p53 acetylation and transcriptional activation (Pearson et al., 2000). HPV-16 E6 has indeed been shown to inhibit the transcriptional activity of CBP/p300 and is able to decrease the ability of p300 to activate p53- and NF-kappaB-responsive promoters (Patel et al., 1999). Moreover, our lab has shown that the E6-p300 interaction is important for the ability of HPV E6 to contribute towards cell transformation, since E6 can complement an adenovirus E1A mutant defective for CBP/p300 binding, in the transformation of primary rodent cells (Bernat et al., 2002).

In the last section of my thesis I provide evidence that E6AP, the E3 ubiquitin ligase utilized by E6 to target p53 for proteasome mediated degradation, is also recruited into PODs in an E6 dependent manner. Overexpressed HPV-18 E6 is mainly localised within the cell in nuclear dots. The absence of good monoclonal antibodies against E6 did not allow me to confidently detect endogenous E6, however, over-expressed E6 resulted in a similar localization in all the cell lines tested (U2-OS [p53+, pRb+], SaoS-2 [p53 -, pRb -], MG13 [p53-, pRb +], HeLa, CaSKi, HaCaT, PML-/- fibroblasts), indicating that the

observed pattern of E6 expression is specific and independent of p53, pRb or PML status. In an attempt to further characterize the proteins present in PODs together with E6, I have shown that several other cellular proteins are recruited by E6. Of particular interest is the fact that E6 has the same dot-like distribution even in a PML null background. This finding is intriguing, since E6 is also able to recruit into these pseudo-PODs, proteins like DAXX and SUMO-1 that are normally present within PODs, but that are dispersed throughout the nucleus in PML minus cells (Zhong et al., 2000a; Lallemand-Breitenbach et al., 2001). This would suggest that E6 acts upstream of PML in the organization of these structures, at least in the case of DAXX and SUMO-1. From the point of view of the viral cycle, this could be a crucial mechanism to control cellular proliferation. PML is in fact not present in PODs upon differentiation, at least in a muscle model system (Kojic S, et al manuscript in preparation), and thus preserving PODs integrity and function might be an essential feature of E6 proteins in helping to maintain cells in cycle even upon differentiation. The enrichment of the proteasome machinery in PODs, induced by E6, was also extremely interesting, and led me to immediately investigate the possibility that these structures were the sites of E6 induced degradation of some of its substrate proteins. E6 targets many cellular proteins for destruction (Scheffner and Whitaker, 2003), via both E6AP dependent and independent mechanisms. Moreover, E6 is itself turned over by the proteasome (Kehmeier et al., 2002). It remains unclear, however, whether E6 is degrading its target proteins in the nucleus or in the cytoplasm. Previous data showed that blocking p53 nuclear export is sufficient to elevate p53 protein levels both in the presence of hdm2 and E6 (Freedman and Levine, 1998). However, p53 protein levels are not completely rescued, still leaving open the possibility that p53 is degraded both in the cytoplasm and in the nucleus. The data presented in this thesis support this model, since both E6 and E6AP can readily assemble in the nucleus, together with the proteasome and other polyubiquitinated proteins.

At the same time the two proteins are also present in the cytoplasm where they are confocal. As mentioned before, p53 regulation involves several post-translational modifications, and an extra level of complexity is provided by its subcellular localization. In this scenario PODs play an essential role, since p53 and several of its cofactors are localised within these structures, where most of its modifications take place (Hofmann et al., 2002b). To define the protein network involved in PML controlled functions, it will be essential to define the dynamics of the spatio-temporal assembly of PODs and, in particular, what controls the transient association of p53 with the different components of these structures.

The presence of PML, E6 and the proteasome in these nuclear dots, suggested to me that PML might exert a negative effect on E6-induced degradation of p53, similarly to what has been recently reported for hdm2 (Louria-Hayon et al., 2003a). The results of my studies clearly show that the presence of PML IV was compatible with E6 mediated protein poly-ubiquitination and degradation of p53. This identifies yet another difference between E6 and hdm2 mediated degradation of p53, other than those already described for the ubiquitination of different lysines within the protein (Camus et al., 2003).

The presence of the proteasome machinery in the nucleus, however, is not something that is exclusively induced by E6, since it has been previously shown that proteasomes colocalise with PML in the nucleus, and that PML itself is turned over through the ubiquitin pathway under physiological conditions (Dino Rockel and von Mikecz, 2002). Moreover a subset of nuclear bodies, termed clastosomes have been found to be enriched for the proteasome machinery as well as for PML (Lafarga et al., 2002).

Making use of a GFP-E6AP expression construct I was also able to demonstrate the presence of the E3 ligase at sites of E6 accumulation in the nucleus. Most importantly I have also shown that a mutant of E6 that is normally excluded from the nucleus is able to

accumulate in dots upon GFP-E6AP over expression. The major implication is that the E6-E6AP complex can migrate and localise in the nucleus where we believe it exerts, at least in part, its degradation functions. The final line of evidence in support of this hypothesis is that p53 is indeed detected in these structures in the nucleus where it is possibly degraded, both in the case of overexpression and also in the case of the endogenous protein. The fact that not all PML foci are positive for p53 staining is not surprising, since not all of them are confocal with E6, and this, once more, raises the possibility that only a specific subset of PML bodies is targeted by E6 and by the proteasome machinery. This is further confirmed by the demonstration that E6AP is recruited to the same PML isoforms as E6, and only when the latter is present.

The overexpression of different PML isoforms is a powerful tool to study the specific functions of each isoform. An interesting observation was that DAXX, a protein involved in multiple apoptotic pathways (Chen and Chen, 2003), is displaced from PODs by PMLV. This is possibly part of the normal regulation of PMLV on DAXX. Similarly it would be interesting to study if other PML isoforms have different effects within the cell, and modulate proteins other than DAXX by affecting their localization, both upon differentiation, as well as upon execution of senescence or apoptosis.

CONCLUSION

As the outermost tissue of the body, the cutaneous epithelium and the mucosal epithelium of the genital and digestive tracts constitute a protective barrier and mediate the interaction with the environment, protecting the organism from water loss and noxious physical, chemical and mechanical insults. The continuous exposure to genetic insults as well as injuries requires that they are continuously renewed throughout life, and these functions are brought about by stem cells that are present in the basal layer and continuously replicate themselves (self-renewal), eventually giving rise to transiently amplifying cells. These cells progressively cease proliferation and initiate terminal differentiation, migrating upwards into the suprabasal layers. Ultimately, keratinocytes produce cornified squames, a complex cytoskeletal architecture of keratin filaments, that are the result of this highly specialized process: epithelial terminal differentiation (Watt, 1989).

This continuous self-renewal has two major consequences for the epidermis. Firstly it makes the epidermis and the mucosa highly regenerative tissues, but at the same time, as for any of the body's stem-cell systems, it has the potential to allow genetic mutations to pass on to the daughter cells in the differentiating tissues, which could eventually degenerate into malignancy. This is an extremely important point to consider, first because of the numerous insults to which these sites are exposed, namely UV, numerous mutagenic agents, mechanical sheer stress as well as the infection of oncogenic viruses like HPV. Secondly it eventually leads to ageing, both extrinsic stress-induced ageing, as well as intrinsic replicative-senescence (Campisi, 1998).

This latter aspect is particularly important, since a well known mechanism that limits clonal growth of malignant cells is cellular senescence. Normal cells have a limited proliferative

life-span, after which they arrest and lose the ability to divide. This phenomenon is termed replicative senescence and is thought to contribute to tissue ageing. The other mechanism that suppresses the growth of undesired cells both during development and in adult tissues, is apoptosis (Vaux and Korsmeyer, 1999). By controlling cell number, apoptosis contributes to essential biological processes, such as morphogenesis. It also occurrs in cells that carry the risk of malignant progression and as well as in response to inflammation is triggered by viruses and by cytokines such as TNF and IFNγ, and thereby constitutes the major cellular anti-tumorigenic defense.

At the same time, since epidermal terminal differentiation results in an irreversible loss of the ability to proliferate and in an ultimately dead structure, it might be considered as a form of senescence, and it has also been proposed to be a specialized form of apoptosis. Although these three processes share some features, they are quite distinct at the molecular level, and this will be discussed below. In light of the experiments presented in this thesis I would like to propose a model in which the HPV E6 and E7 proteins may regulate all three processes, by targeting a key nuclear compartment: the PML Nuclear Bodies (Summarized in **Figure 39**).

First I would like to briefly analyse differences and similarities between the three processes.

Differentiation and Apoptosis.

Epidermal terminal differentiation and apoptosis are both metabolically active processes that provoke a series of cellular modifications and ultimately lead to cell death. During apoptosis the nucleus condenses and the DNA is fragmented by endonucleases in a characteristic pattern, an event that can be detected by in situ dUTP-biotin nick end labelling (TUNEL). TUNEL positive keratinocytes have been consistently found in the granular layer of the epidermis in differentiating cells before cornification. The execution

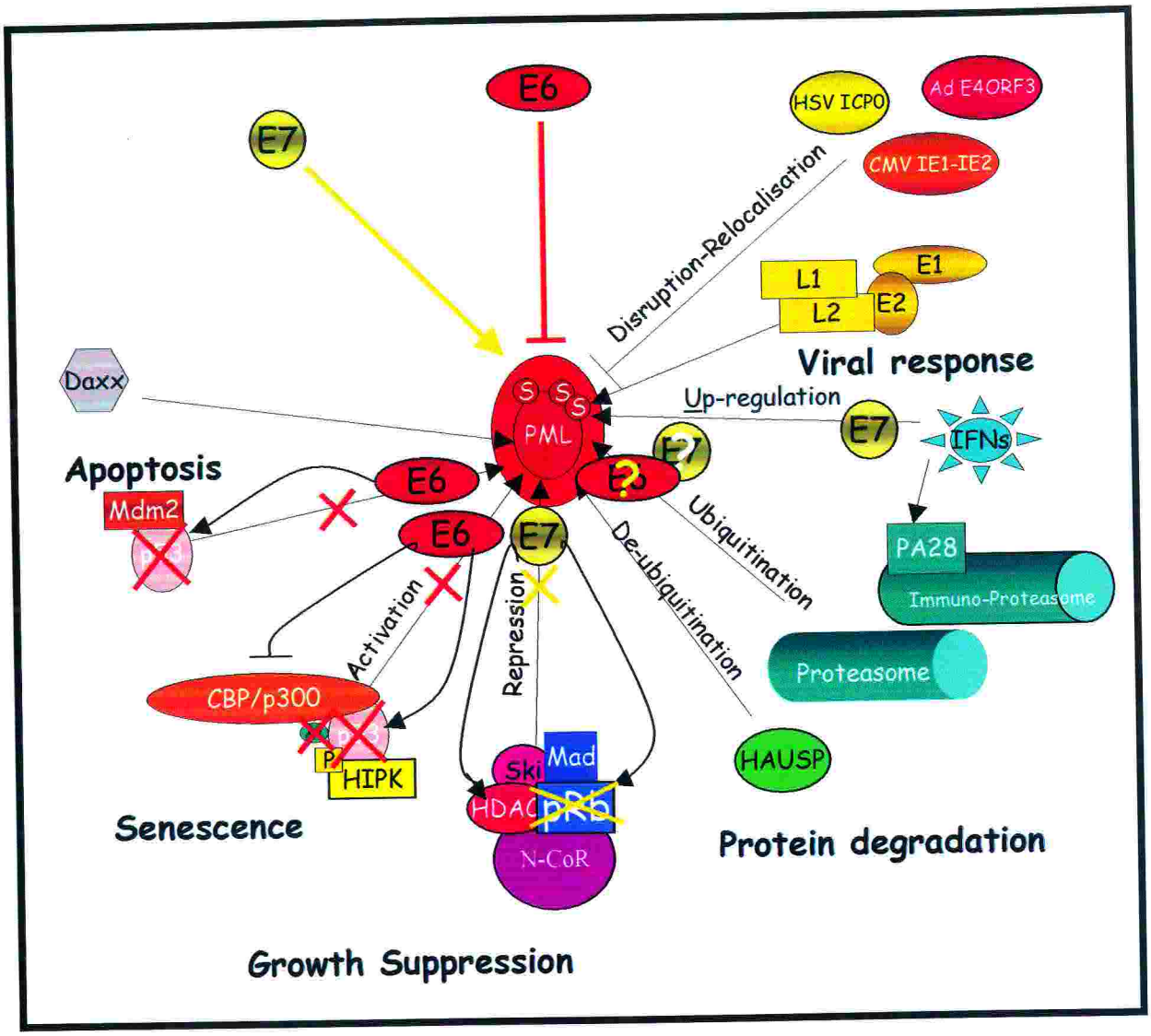

Fig. 39 Model of how the HPV E6 and E7 oncoproteins may perturb the role and function of PML interacting proteins. PML has a central role in regulating apoptosis, senescence, growth suppression, protein degradation and the anti-viral response. Abbreviations: APL (Acute Promyelocitic Leukaemia), S (SUMO-1), P (Phosphorylation), Ac (Acetylation).

of apoptosis is brought about by a family of proteases termed caspases (Vaux and Korsmeyer, 1999) and during keratinocyte terminal differentiation most cellular components are degraded, organells are lost and all of this probably occurs by proteolysis. Differences however have been found in the pattern of proteins that are specifically cleaved during the execution of these two processes, and partial digestion of spectrin during terminal differentiation of lens fiber cells is one example of this (Lee et al., 2001). In HPV expressing cells, the constant expression of the E6 protein inhibits apoptosis, mainly by degrading the p53 tumour suppressor (Scheffner et al., 1990). However in transgenic mice, E6 and a p53-null genotype act additively to reduce levels of apoptosis induced by E7, indicating that E6 modulates apoptosis at least in part through p53-independent mechanisms. One of the main p53-independent effectors of apoptosis is Bak, a member of the Bcl-2 family, and that has been demonstrated to be bound and degraded by E6 through the proteasome pathway (Thomas and Banks, 1999; Jackson et al., 2000). Regulators of apoptosis, such as the pro apoptotic Bak, Bax, as well as the anti-apoptotic Bcl-2 and Bcl-xL, have also been involved in epidermal differentiation, and are differentially expressed in keratinocytes (Rodriguez-Villanueva et al., 1998; Delehedde et al., 1999). Bcl-2 has been found associated with proliferative keratinocytes in normal skin and basal cell carcinomas(Delehedde et al., 1999). Conversely, Bax and Bak are up-regulated during differentiation, reaching their maximum within the granular layer, and Bcl-xL expression increases in suprabasal layers in some skin diseases (Krajewski et al., 1996). It is thus possible that the interaction between E6 and Bak is also important to modulate terminal differentiation. It is in fact essential to underline the importance for HPV to maintain the infected cell in a metabolically active state, in order to complete the viral life cycle, but at the same time to maintain a certain degree of differentiation, since the major viral promoter is regulated by tissue and differentiation specific factors (Hoppe-Seyler et al., 1991;

Bauknecht et al., 1992; Andersen et al., 1997; Ai et al., 2000). Indeed, early studies, using an in vitro raft culture system, demonstrated that E6-E7 immortalised cells alter the overall organization of the epithelium, with parabasal cells extending throughout most of the sections, and with abnormal nuclei present in the upper regions. Examination of E6-E7-expressing cell lines in the raft system at a later passage revealed that complete loss of morphological differentiation had occurred (Hudson et al., 1990).

An extremely interesting interaction from this point of view is the one between E6 and the transcription factor c-myc (Gross-Mesilaty et al., 1998). c-myc expression induces apoptosis in a variety of cell types when deprived of growth factors, otherwise it promotes cell cycle progression (Packham and Cleveland, 1995), while it interestingly promotes differentiation of epidermal stem cells. Thus, activation of c-myc for 5 days in keratinocytes was demonstrated to provoke a dramatic increase of stratification, cell size and expression of involucrin (Gandarillas and Watt, 1997). At the same time it is important to keep in mind that during keratinocytes differentiation, Mad, a c-myc binding partner and antagonist, is rapidly induced and c-Myc is downregulated (Ayer et al., 1993). During HPV infection high-risk E6 and E7 subvert this equilibrium, and c-myc levels remain elevated in all basal-like cells, while Mad levels decrease (Hurlin et al., 1995). Interestingly Mad repressive effects have been associated with PODs, and PML has been shown to be essential for Mad repressor activity (Khan et al., 2001a). The mechanism seems to involve the interaction with major co-repressor complexes such as the c-Ski, N-CoR, mSin3A and HDAC1 containing complexes (Jones and Shi, 2003; Thiagalingam et al., 2003). It is tempting to speculate that E6 mediated deregulation of PML functions may help the cell to escape from the Mad-mediated differentiation stimulus, in favour of cell cycle progression. Moreover E6 and c-myc have been shown to cooperate to activate the h-TERT promoter, further linking E6 presence in PODs to essential functions leading to cell immortalization

(Veldman et al., 2003).

As shown in this thesis, E7 is also linked to PODs, but this does not seem to involve the pRB-binding LXCXE motif. Interaction with the Skip/Ski complex (Prathapam et al., 2001) as well as directly with the NURD Mi2beta/HDAC1 containing complex (Brehm et al., 1999), may be thus involved and this could lead to a transient alteration of the equilibrium of such complexes, activating genes that are under Mad repression, such as tyrosine phosphatase cdc25A (Shinagawa et al., 2000), as well us under the control of other co-repressors. A recent report did indeed demonstrate that HPV-16 E7 elevates cdc25A levels (Nguyen et al., 2002a). This phosphatase plays an essential role in regulating the G1/S transition, and E7 by targeting PODs may well thus impede growth arrest induced during serum starvation, as well as keratinocyte differentiation, in which cdc25A plays a crucial role in both processes (Amati et al., 1998). Importantly, such activation seems to involve the distal E2F site within the cdc25A promoter, pointing at the HDAC1 interaction as the crucial one in this particular case (Nguyen et al., 2002a).

Going back to the comparison between apoptosis and terminal differentiation, one of the crucial players in both processes is the p53 tumour suppressor (Wolkowicz and Rotter, 1997). p53 triggers cell cycle arrest in DNA-damaged cells allowing the repair process to occur. Indeed, p53 itself or p53-regulated proteins enhance Nucleotide Excision Repair (NER), the major mechanism by which UV damage is repaired in the skin, and it does so in basal undifferentiated keratinocytes, but not in the upper differentiated layers (Li et al., 1997). Moreover, there are data showing that p53 is involved in regulating the expression of cytokeratins, such as K19 (Moles et al., 1994). p53 maximum expression is found in proliferating keratinocytes, while in the transition from proliferation to differentiation p53 is down-regulated (Dazard et al., 2000). p53 plays a role in normal, undamaged, epidermis, since p53 null keratinocytes have an accelerated malignant progression when they are

transfected with v-ras (Yuspa et al., 1994). Moreover p53 null mice have demonstrated that a normal p53 gene is dispensable for embryonic development, but its absence renders the animal prone to the development of multiple neoplastic diseases (Donehower et al., 1992). There are however clear differences between epidermal differentiation and apoptosis, both at the cellular and molecular levels. Perhaps the two most striking are firstly that rather than shrinking like in apoptotic cells, differentiating keratinocytes become bigger and flatter. Secondly, keratinocytes can undergo terminal differentiation without a significant presence of apoptotic changes (Gandarillas et al., 1999). These differences are exemplified at the molecular level by the difference played in these two processes by two major players, such as p53 and c-myc (Gandarillas and Watt, 1997; Dazard et al., 2000). In the case of HPV-infected cells it seems that the expression of E7 can force differentiated cells to enter S phase, but this is then followed by either endoreduplication, resulting in 4n or higher ploidy, and is not followed by cell division (Chien et al., 2002) or alternatively by p27kip1, cyclin E, and p21cip1 accumulation, resulting in cell cycle arrest and preventing further S-phase re entry (Chien et al., 2002). Similar results were obtained in a muscle differentiation system, where E7 expression allowed DNA synthesis during and after differentiation. In terminally differentiated myotubes, instead, it failed to reactivate DNA synthesis even though it was shown to be functional in the system used. When expressed in myoblasts, despite the high cyclin E protein levels induced by E7, the myotubes remained devoid of cyclin E-associated kinase activity, probably due to the elevation of CKIs such as p27 (Sacco et al., 2003). In light of what I have mentioned before and of the results presented in my thesis, it is clear, how critical the control of all these processes is to growth and differentiation, and how targeting PODs structures can be seen to be crucial for the HPV life cycle. C-myc may well be the major coordinator of these processes since it induces the sequestration of p27 in a form in which it can no longer inhibit cyclin E/CDK2 activity

(Vlach et al., 1996), a process in which E6 might cooperate.

Differentiation and Senescence.

As for the relationship between terminal differentiation and senescence, there are clearly similarities as the upregulation of cyclin dependent kinase inhibitors p16, p21, and p27, and the down regulation of cyclin A, cdk2, or cdc2, even though differentiation is a rather active process and needs other positive stimuli in order to occur (Hauser et al., 1997; Harvat et al., 1998). Experiments using in vitro keratinocyte cultures have demonstrated that differentiation could be induced at all passages by raising the calcium concentration in the medium. Differentiation could also be induced in senescent cultures, however the potential to undergo differentiation decreased as keratinocytes aged in culture, pointing at the execution of two separate pathways (Norsgaard et al., 1996). Thus, cellular senescence is not linked to epidermal differentiation. From the viral point of view, however, it remains crucial to avoid the execution of both pathways.

One of the key players in controlling cellular senescence is the catalytic subunit of cellular telomerase (hTERT). It is the imperfect replication of chromosomal ends that is, in fact, one of the main reasons to explain how stem cells lose their potential for self renewal (Counter, 1996). Each cell division indeed causes shortening of telomeres, which eventually reach a critical length and beyond this threshold they become unstable, and cells senesce. The length of telomeres is controlled by telomerase, which is expressed in immortalized cells, in tumor cells, and in the basal layer of normal epidermis (Harle-Bachor and Boukamp, 1996), as well as in other stem cells (Kim et al., 1994), but not in most normal, adult cells. Both HPV E6 and E7 proteins are involved in deregulating this key cellular regulatory mechanism acting via c-myc and the p16/Rb pathways, and both pathways use PML Nuclear Bodies as central organizing centers (Kiyono et al., 1998; Veldman et al., 2003).

In conclusion these three processes, differentiation, apoptosis and senescence are distinct from each other both spatially ad temporally. In keratinocytes, the differentiation schedule is executed as cells move up in the epidermal layers to reach a final squamous state that protects the organism from external insults. Apoptosis however is instead executed in response to certain situations that cause DNA damage or in the presence of certain pathogens, and results in the elimination of the damaged cell. Finally senescence is part of the normal process that reduces stem cell self-renewal capability, resulting in skin-ageing. Nonetheless all three processes share key molecular components and most importantly, common structures within the nucleus, PML Nuclear Bodies, that allow integration of all these different stimuli. This thesis provides evidence that these sites are targeted by two HPV proteins, E6 and E7, and that this allows them to impair cellular senescence, to modulate and degrade p53, thereby modulating apoptosis and possibly differentiation.

MATERIALS AND METHODS

Plasmids.

For *in vivo* expression:

E7: HA-tagged HPV11 E7 (**HA-11E7**), and HPV16 E7 (P.Massimi) (**HA-16E7**) were cloned into a PCDNA3 backbone (Invitrogen) by standard PCR methods (BamHI-EcoRI sites), with the HA tag at the N terminus.

This was done using the following pair of primers:

Forward HA11E7 (BamHI) : ATAATAGGATCCATGCATGGAAGACTTGT

Reverse HA11E7 (EcoRI): ATAGAATTCTTATGGTTTTGGTGCGCAGATG

The resulting constructs were then verified by DNA sequencing.

E6: HA-tagged HPV11 E6 (**HA11E6**) and HPV18 E6 (**HA18E6**) in the GWI expression vector have been kindly provided by Ron Javier. GWI is a CMV-based expression vector (GWI, British Biotechnology, Oxford, United Kingdom).

The HA-tagged HPV18 E6 construct expressing just the full length protein (**HA18E6NS**) was obtained using the "Genetailor site directed mutagenesis system" (Invitrogen) according to the manufacturers instructions. A G to C mutation was inserted at the splice donor site (position 130 from ATG) as described previously for HPV-16 E6 (Sedman et al., 1991). The two NLS mutants were obtained by substituting the KRR at position 124-126 with AAA (NLS1 mutant) using the "Genetailor site directed mutagenesis system", and the RR at position 153-154 with GG (NLS2 mutant) by standard PCR methods.

Genetailor site directed mutagenesis primers were as follows:

Forward HA18E6 NS: CAGTATTGGAACTTACAGAGCTATTTGAATTTC

Reverse HA18E6 NS: CTCTGTAAGTTCCAATACTGTCTTGCAATAT

Forward HA18E6 NLS1:

AACTTAGACACCTTAATGAAGCGGCGGCGTTTCACAACATA

Reverse HA18E6 NLS1: TTCATTAAGGTGTCTAGTTTTTCTGCTGG

PCR primers

Forward HA18E6 NLS2: ATAATA AGATCT ATGGCGCGCTTTGAG

Reverse HA18E6 NLS2:

ATAATA GAATTC TTATACTTGTGTTTCTCCGCCTTGGAGTCG

The **HA-E6*I** construct was obtained by cloning the E6*I cDNA into a PCDNA3 backbone (Invitrogen) by standard PCR methods (HindIII-EcoRI sites).

pcDNA3 18 E6 ΔA (47-49aa) has been previously described (Pim et al., 1994). The resulting constructs were then verified by DNA sequencing.

PML: As part of a collaborative work plasmids expressing **FLAG- PML isoforms I-VI** in **pCIneo** were kindly provided by Katherine J. Lethbridge, Neil Killick and Keith N. Leppard; Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK.

FLAG-PML clones are in a pCIneo backbone (CMV-based expression vector, Promega, T7 promoter for in vitro translation use), based originally around a clone for PML VI that had the cDNA inserted with NcoI and EcoRI ends into the vector SmaI site (kindly provided by R. Everett). This was modified with a FLAG oligo at the N-terminus between vector EcoRI and SalI sites to give a 12 residue-N-terminal extension carrying the epitope. Different isoforms were then made by substituting-in the relevant C-terminal fragments

(cDNAs kindly provided by E. Solomon, P. G. Pelicci and A. Dejean) from the SmaI site in common exon 5 and the products checked by sequencing.

Other plasmids: PCDNA3-p53 was available in the lab and has previously been described. PCDNA3-DAXX was kindly provided by G.Del Sal.

GFP-E6AP was cloned by standard PCR techniques into a standard EGFP vector. (Kan resistence, CMV promoter, Clontech). The product was checked by sequencing.

For *in vitro* expression: **pCIneo FLAG-PML I-VI** were translated from the T7 promoter present at the 5' end of all FLAG constructs;

For GST-pull down assays: pGEX2T.11E6, pGEX2T.18E6, pGEX2T.11E7 and pGEX2T.16E7 were available in the lab and have previously been described.

Antibodies

The following antibodies were used in this study:

Primary antibodies: anti-**HA rat** monoclonal (3F10, Roche WB: 1:500); anti-**HA mouse** monoclonal (12CA5, Roche IF: 1:100), anti-**HA rabbit** polyclonal (Y-11, Santa Cruz IF: 1:200), **anti-α-actin**, (rabbit polyclonal, Sigma WB: 1:1000), anti-**p53** mouse monoclonal antibody (DO-1, Santa Cruz IF: 1:200 WB:1:1000), anti-**PML mouse** monoclonal antibody (PG-M3, Santa Cruz IF: 1:100), anti-**PML rabbit** polyclonal antibody (H-238, Santa Cruz IF: 1:200), anti-**Flag** mouse monoclonal antibody (M2, Sigma 1:1000 IF:1:1000 WB:1000), anti-**Daxx** rabbit polyclonal antibody (M-112, Santa Cruz IF: 1:200), anti-**cyclin B1** mouse monoclonal antibody (Santa Cruz WB: 1:1000), anti-**GFP** rabbit polyclonal antibody (Living color peptide, ClontechWB: 1:200), anti-**poly ubiquitin** mouse monoclonal antibody (FK2, Affinity IF:1:200), anti-**proteasome** mouse monoclonal antbody (S20, Affinity, IF:1:200), anti-**SUMO-1** mouse monoclonal antibody (GMP1,

Zymed, IF: 1:200), anti-**hDlg** mouse monoclonal antibody (2D11, Santa Cruz IF: 1:50), anti-**Scribble** goat polyclonal antibody (C-20, Santa Cruz IF: 1:50), anti-**cytochrome c** rabbit polyclonal antibody (H-104, Santa Cruz IF: 1:200).If not otherwise stated, these were used in WB at 1:1000 or 1:500 dilution and 1:200 or 1:100 for immunofluorescence analysis. Secondary antibodies were used as follows: anti-mouse-HRP, (Sigma 1:2000); anti-rat-HRP (DAKO 1:500); anti-rabbit-HRP (Sigma 1:2000), rhodamine conjugated goat anti-rabbit, (Molecular Probes 1:1000) and fluorescein conjugated goat anti-mouse, (Molecular Probes 1:1000).

GST fusion protein expression and purification

For protein production and purification, the appropriate expression plasmid was freshly transformed into *E. coli* strain BL-21 and after an overnight incubation a polyclonal pool of colonies was diluted in Luria Broth containing ampicillin and grown at 37°C up to an OD of 0.5. Recombinant protein expression was induced for five hours with 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (20mM Tris-HCl pH 7.5, 250mM NaCl, 0.5% NP40, 2μg/ml aprotinin, 100μM TPCK and 50μM TLCK, 1mM PMSF) and the lysates were then cleared from cell debris by centrifugation at 17000 rpm. The GST-fusion proteins were then incubated for one hour at 4°C with glutathione-conjugated agarose beads (Sigma) in lysis buffer and bound proteins were washed three times for 10' at 4°C. A final wash was performed in cold PBS to eliminate residual NP40 and then the proteins were stored in 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 2.5 mM EDTA 20% glycerol at -20°C until used. The levels and purity of proteins were determined by SDS-PAGE and subsequent Coomassie Brilliant Blue R (Sigma) staining.

In vitro transcription-translation and GST pull-down assays

Proteins were expressed in vitro, using the TNT reticulocyte lysate system (Promega) in the presence of L-^{35}S-labeled cystein (Amersham 0.6μCi/1μl of reaction volume) for 2 hours. This was then incubated for 90' at room temperature with equal amounts of GST fusion proteins bound to glutathione-linked agarose beads. The reaction was carried out in a final volume of 50μl, in a binding buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 2.5 mM EDTA. Bound proteins were washed extensively in PBS/0.5%NP40. SDS-PAGE and autoradiography were used to analyze the pattern of bound proteins. Assays were quantitated using a phosphoimager (Instant Imager, Packard).

Cells and Transfections.

U2OS (human osteosarcoma cell line p53+, pRb+), SaOS-2 (human osteogenic fibrosarcoma cell line, p53-, pRb-), MG63 (human osteosarcoma cell line p53-, pRb+), CaSki (human cervical epidermoid carcinoma cell line, HPV16 positive), HeLa (human cervical carcinoma cell line, HPV18 positive), HaCaT (immortalized human skin keratinocytes), PML-/- fibroblasts (kindly provided by P.P. Pandolfi and G.DelSal (Wang et al., 1998a)), BRK (baby rat kidney cells) obtained from 9day-old Wistar rats were all grown in DMEM plus 10% heat inactivated foetal calf serum. Transfections were done by the calcium phosphate precipitation method as described previously (Wigler et al., 1979) or by SuperFec reagent (Qiagen) as indicated by the manufacturer. For the proteasome inhibition experiments cells were grown for 2 hours in the presence of 50μM N-CBZ-LEU-LEU-LEU-AL (MG132) diluted in DMSO (Sigma) or with DMSO alone as a negative control.

Cells were plated in 6-well-dishes ($1x10^5$ per plate) for WB analysis, on 10-cm dishes for

immunoprecipitation analysis ($5x10^5$ per plate) and directly onto 4 well or 8 well coverslips (Nalgene Nunc) for immunofluorescence analysis ($4.5x10^4$ per well in the case of the 4 well slides, and 2.25 $x10^4$ per well in the case of the 8 well slides). 0.2 μg of the E6 and E7 expression plasmids, 0.05 μg of the FLAG-PML constructs and 0.1 μg of the Daxx plasmid, were used in the immunofluorescence experiments. 0.5 μg of PML plasmids, 2.5 μg of the E6 plasmids, and 0.1 μg of GFP plasmid were used in the immunoprecipitation and western blot experiments.

Cell cycle analysis

Cells were synchronized by a single aphidicolin block. The drug at a final concentration of 5μg/ml was left 24 hours and the release was obtained by washing three times with pre-warmed medium. After 5 h cells were synchronized in S phase and harvested. After 9-10 h, depending on the cell line, cells entered M phase. Standard FACS analysis was carried out as follows. Cells were trypsinized and fixed in 70% EtOH. The pellet was then resuspended in a 50μg/ml propidium iodide/0.013%NP40/130μg/ml RNAse/ 0.1%Sodium Citrate solution and left for 15 minutes in the dark, at room temperature. Data were then collected using a Becton Dickinson FACSCalibur machine and analysed using the Cell Quest programme.

Immunofluorescence.

24h after transfection U2OS and CaSKi cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature, washed once with 0.1 M glycine, permeabilized with 0.1% Triton X-100 in PBS and then incubated with the primary antibody diluted 1:200 for 1h in PBS 2% BSA. HA-tagged proteins were detected with a polyclonal rabbit antibody (Y-11, Santa Cruz 1:200), p53 with a mouse monoclonal antibody (DO-1, Santa Cruz 1:200),

PML with a mouse monoclonal antibody (PG-M3, Santa Cruz 1:100), Flag-tagged proteins with a mouse monoclonal antibody (M2, Sigma 1:1000), Daxx with a polyclonal rabbit antibody (M-112, Santa Cruz 1:200). Following five washes in PBS, the cells were incubated with a 1:1000 dilution of fluorescein conjugated goat anti-rabbit antibody (Molecular Probes) and Rhodamine-Red conjugated goat anti-mouse antibody (Molecular Probes) for 20 min. The cells were then washed several times in water and mounted on glass slides using Vectashield mounting medium (Vector Labs Inc.). Experiments using different secondary antibodies in dual labeling experiments or using secondary antibodies in the absence of the primary, were performed to check for artifactual results.

Confocal Microscopy

Slides were analyzed using a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480nm and 510nm. The data were collected at 1024x1024 pixels resolution. The microscope was a Zeiss Axiovert 100M utilizing a 100x objective oil immersion lens. The scanning conditions used were kept constant in each experiment and ensured that the signal overlap between channels was essentially eliminated.

Immunoprecipitations and western blotting

U2-OS cell were transfected and harvested 24h later. Cells were washed twice in cold PBS, then scraped in high-salt E1A buffer (25mM Hepes pH 7.0, 0.1%NP40, 500mM NaCl, protease inhibitors cocktail, phosphatase inhibitor and 1mM NaButyrate). Cells were left on ice for 20 min and then sonicated for 5 seconds. The pellet (insoluble fraction) was separated from the soluble fraction (supernatant) following centrifugation at 13K rpm.

Extracts were subjected to SDS PAGE and western blotting onto a 0.22 μm membrane (Schleicher and Schuell) and then incubated first with the appropriate primary antibody

(specified in the text) overnight at 4 °C: rat monoclonal 3F10, (Roche 1:500), was used to detect HA-tagged proteins, rabbit polyclonal GFP living colors, (Clontech 1:1000), to detect GFP, rabbit polyclonal anti-actin, (SIGMA 1:1000), to detect actin, mouse monoclonal anti-cyclin B1, (Santa Cruz 1:1000), to detect cyclin B, mouse monoclonal anti-Flag M2 (SIGMA 1:1000) to detect Flag tagged PML, rabbit polyclonal anti-PML H-238 (Santa Cruz 1:500) to detect both endogenous and overexpressed PML. All of the antibodies were used in 1% milk/PBS, then washed three times in 0.2% Tween/PBS and further incubated 1h at RT with the peroxidase conjugated secondary antibody. After extensive washings with 0.2% tween/PBS protein levels were assessed through a standard ECL reaction (Amersham) or a femto-ECL (Pierce) in the case of HA-E6.

For immunoprecipitation experiments, the soluble fraction was diluted down to a final salt concentration of 275 mM NaCl and then incubated for 3-4 hours with the appropriate antibody (0.5 μg) on a rotating wheel at 4°C. The immunocomplex was then precipitated by adding 20 μl of protein A/protein G 50% slurry (Amersham Pharmacia Biotech), prewashed in 275 mM NaCl. After a 60 mins incubation the beads were spun down and washed six times in 275 mM NaCl E1A buffer. The beads were finally boiled and subjected to SDS PAGE followed by western blotting.

PML induction of senescence in BRKs

Primary BRK cells were obtained from the kidneys of 9-day old Wistar rats. The tissues were first washed several times in DMEM without serum, then homogenized and finally trypsinised using 0.25% Trypsin solution in PBS. After three steps of trypsinisation, 15 minutes each, at 37°C, the reaction was blocked with 50% FCS. The cells were then centrifuged for 10 minutes at 12000rpm and the pellet was resuspended in supplemented DMEM. From one rat enough cells were obtained for plating 6-8 10 cm tissue culture

dishes. After two days in culture the BRK cells were co-transfected with an EJ-ras oncogene as a positive control or with PML isoforms IV and VI alone or in combination with HPV-11 E6 or HPV-18 E6. Cells were kept under G418 selection (2.28 μg/ml) for 12 days (Genticin, GibcoBRL) and then fixed with formaldehyde (3,7% solution, Merck), and stained as follows for Senescence Associated β-Galactosidase (SA-β-Gal) (previously described by (Dimri et al., 1995)). Cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature, washed in PBS and incubated at 37°C (no CO2) with fresh SA-β-Gal stain solution (X-Gal 1 mg/ml, 5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 150mM NaCl, 2mM MgCl, 40 mM Citric Acid/sodium phosphate pH 6.0). Staining was maximal after 10-12 hours. The number of blue cells were then scored under a light microscope.

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