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Alteration of p53 and NF- κ B pathways by E7 protein from cutaneous Human Papillomavirus type 38

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TITRE

**Alteration of p53 and NF- κ B pathways by E7 protein from
cutaneous Human Papillomavirus type 38**

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Chapter I: General introduction

Short history of papillomavirus-induced carcinogenesis

Human papillomaviruses (HPV) are small non-enveloped double-stranded circular DNA viruses. Their genome is approximately 8 kb and is incorporated in an icosahedral capsid (Figure 1) (1). HPVs are epitheliotropic viruses that infect keratinocytes at a wide range of body sites. To date, based on their DNA sequence variation, 170 HPV types are identified (2). The most frequent manifestation of HPV infection is the development of warts. However, some HPV types are involved in the development of cancers. The research in HPV field is centered on the study of the relationship between HPV infection and cellular transformation.

Research on papillomaviruses (PV) was initiated about more than a century ago and passed through several phases. Few years after the use of filters retaining bacteria to demonstrate the filterability of the causative agent of tobacco mosaic disease (3), in late 19th century, experimental transmission of papilloma in dogs was reported (4). These were the first observations suggesting viruses as causative agents. In 1907, Ciuffo reported the cell-free transmission of human warts, demonstrating for the first time a viral etiology for human disease (5). A second phase of research in this field was stimulated by several observations related to the biology of PV in animals, in the 1930s. This period was initiated by the study of cottontail rabbits' papilloma in 1933. Extracts isolated from large warts of wild cottontail rabbits' lesions contained infectious particles capable of inducing cutaneous papilloma in domestic rabbits (6). This virus is now known as the Cottontail rabbits PV (CRPV) and became an important experimental model of viral tumorigenesis. With the advent of electron microscopy, tobacco mosaic virus was observed first in 1939 and a decade later HPV particles visualization was achieved (7). Although the research on papillomaviruses was hampered by the inability of these viruses to propagate *in vitro*, the interest on this field increased when Ito and Evans showed that papillomavirus DNA inoculation into rabbits was sufficient to induce carcinomas (1961) (8). This was the first demonstration that PV genome displays oncogenic properties. Later on, Crawford and Crawford elucidated the physical properties of HPV DNA, in 1963 (9). Following the revolution of molecular cloning in the 1970s, cutaneous HPV types were identified in malignant skin lesions in patients with a rare hereditary skin disorder, *Epidermodysplasia Verruciformis* (EV) (10), providing the first link between HPV and human cancer. With the use of the new molecular biology technics, the plurality of HPV types was

demonstrated and two mucosal low-risk HPV types (i.e. 6 and 11), and shortly after, two mucosal high-risk HPV types (i.e. 16 and 18) were isolated from genital warts in early 1980s (11-17). The initial studies aiming to demonstrate the role of HPV in human carcinogenesis was based on the hypothesis that only one type existed. This assumption led to many contrasting results when different studies analyzed lesions from different anatomical regions. The discovery of the existence of different HPV types with mucosal or cutaneous tropism explained the inconsistencies reported in many studies. Most importantly, these new findings overcame the criticism of the implication of HPV in cervical cancer proposed in 1976 (18), and epidemiological study clearly identified HPV infection as main risk factor for cervical cancer (19). In 1987, studies of Durst and colleagues showed *in vitro* experimental models the oncogenic properties of mucosal high-risk HPV16, and in particular highlighted the transforming abilities of the products of two early genes, E6 and E7 (20). Subsequently in last three decades, a vast number of studies have fully characterized the biological properties of E6 and E7 as main viral oncoproteins, being able to deregulate fundamental cellular events, such as cell cycle and apoptosis (21). Thanks to the knowledge built on HPV biology, prevention methods are developed to defeat cervical cancer (22-24).

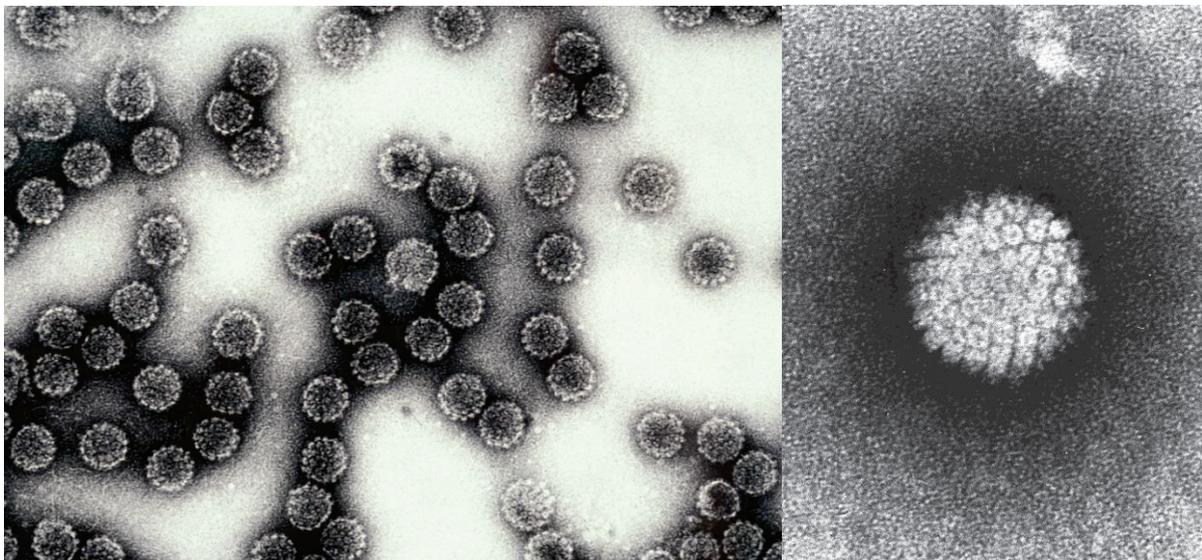


Figure 1. Electron micrograph of negatively stained papillomaviruses.

Sources: left picture from Inserm unit 190, right picture from NIH-Visuals Online n° AV-8610-3067.

Several screening strategies for HPV-induced cervical lesions have been put in place in many worldwide countries, determining a strong decrease of cervical cancer incidence. In addition, two prophylactic HPV vaccines are now commercially available, one quadrivalent that included two mucosal low-risk HPV (e.i.6 and 11) and two mucosal high-risk HPV (e.i.16 and 18) and one bivalent that includes two mucosal high-risk HPV (e.i.16 and 18).

Although research was initially focused on the role of mucosal high-risk HPV types in cervical cancer, it is now well established that this group of viruses are the etiological factor of a subset of other genital tract cancers (e.g. vagina, vulva, anus, and penis) as well as head and neck cancers (25-29). HPV research is still expanding the aim to identify new links with additional human cancers; such as cutaneous HPV types and nonmelanoma skin cancer (NMSC) (30, 31).

Classification and phylogeny

The HPVs were originally included in the *Papoviridae* family, together with polyomaviruses. Subsequently, due to morphological characteristics and the accumulated knowledge on PVs genome, a distinct family of *Papillomaviridae* was created. PVs are classified according to the species of origin and the degree of relationship between viral genomes. A recent phylogenetic tree has been designed groups the different PV types and at least 170 HPV types are presently identified (Figure 2) (32, 33). The open reading frame of L1 gene is particularly conserved among PVs and is used for their classification. Phylogeny of PVs is organized with following taxonomic levels: family, genus, species, types, sub-types, and variants (32). *Papillomaviridae* family is composed of 16 genera with less than 60 % homology (from alpha to pi). Each genus is divided into several numbered species (60 to 70 % homology) that are divided into types (71 to 89 % homology). Subtypes differ with only 2 to 10 % and variant with less than 2 % differences (34). Variants are indicated in reference to a prototype sequence of a PV gene and one nucleotide variation can be sufficient to define a variant.

HPV are represented by the 5 genera: alpha, beta, gamma, mu and nu. Based on their tissue tropism, two main groups of HPV have been identified: cutaneous and mucosal. According to their oncogenic potential, mucosal HPV types are divided into low- and high-risk. Some of the

cutaneous types are preferably found in EV associated with a high risk of developing skin cancer upon UV-exposure.

Morphology and genomic organization

The HPV viral particle is approximately 55 nm in diameter and formed by an icosahedral capsid that is composed of L1 and L2 proteins arranged in 72 capsomers (Figure 1) (35). The number of L1 and L2 proteins was investigated and recent studies report that the capsid of HPV is composed of 360 L1 and 72 L2 molecules (36, 37). L1 plays an important role in the formation of the capsid and L2 is mainly involved in the encapsidation of the viral genome (38).

The HPV genome is organized as illustrated in figure 3. The genome size is approximately 8 kb forming a chromatin-like complex by associating histones. It is divided in three regions: (i) the long control of about 1 kb, (ii) the early region of about 4 kb, and (iii) the late region of about 3 kb. The long control region (LCR) encodes the *cis* regulatory elements regulating replication and gene expression (1, 39). The early region contains genes expressed during the initial phase of the virus life cycle and their products are important in genome replication and transcription as well as in processes of host cell transformation. The late region encodes the capsid proteins that are involved in the last steps of the virus life cycle. Transcription of each of the coding regions, early and late, is regulated through its respective promoter containing responsive elements for host-cell transcription factors. In undifferentiated cells only the early promoter is active whereas both early and late promoters are active in differentiated cells (40, 41).

Despite the highly conserved structure of the genome, HPV types of different genera have some exclusive features. For instance, the beta HPV genome is relatively short in comparison to the mucosal HPV types, ranging from 7.4 to 7.7kb. This fact is due to the considerably reduced size of the LCR, around 400bp compared to 650-900bp in other papillomaviruses. Beta HPV types display some differences also in the coding region. Most of them lack the E5 gene, with the only exception of HPV14. In addition, the E2 ORF of all the beta HPV types is much longer compared to the other papillomaviruses. Also the mucosal high-risk HPV types present some specific characteristics. Indeed, HPV 16, 18 and 31 produce an additional early protein,

E8^{E2C} that represses the expression of the viral oncogenes E6 and E7 and consequently cellular proliferation (42).

Similarly to beta HPV genus, gamma HPV types lack the E5 gene. The isolation of three novel and related gamma HPV types (101, 103 and 108) from cervical tissues has been recently reported. Interestingly, the genomes of these three gamma HPV types lacks the E6 open reading frame (43, 44).

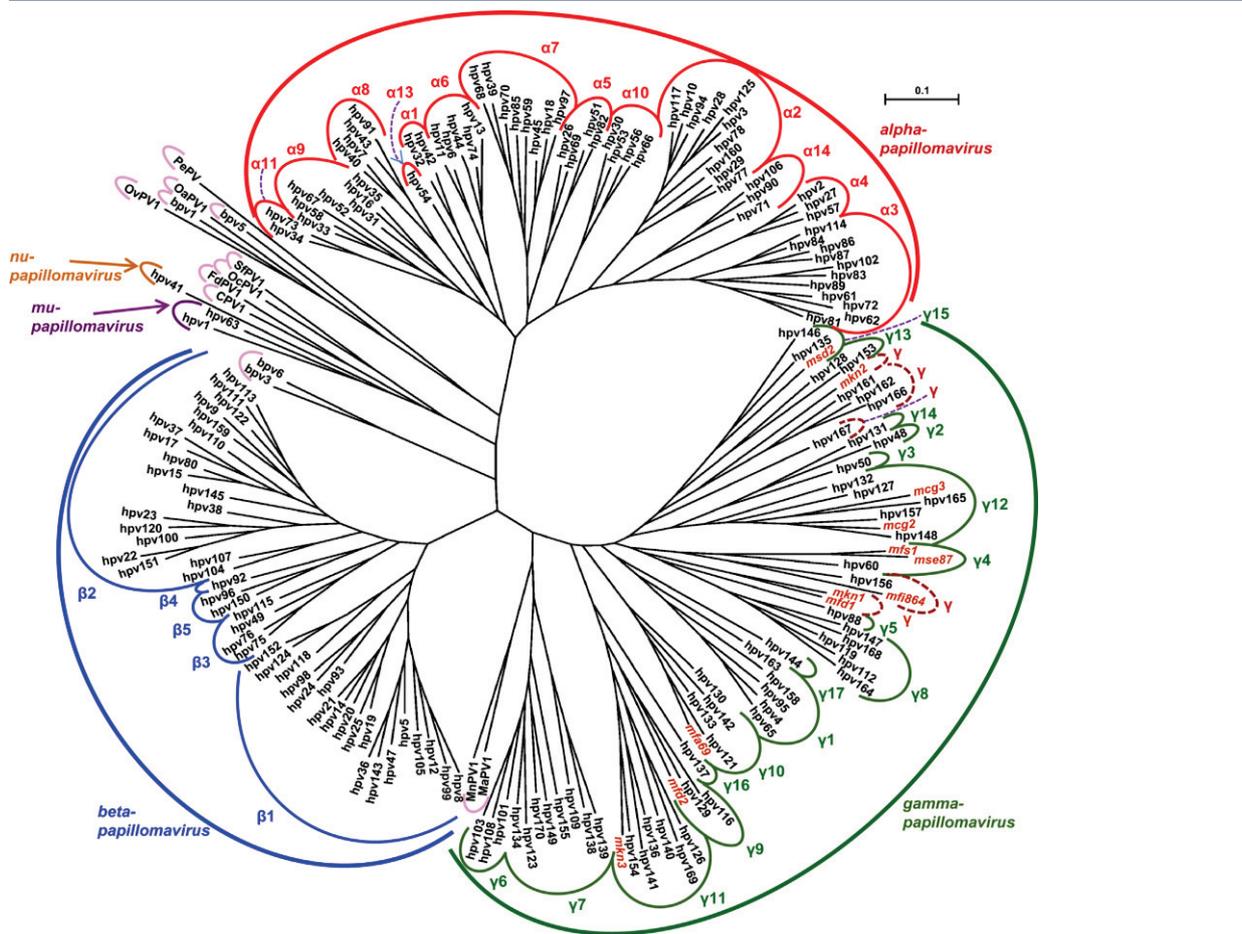


Figure 2. Papillomavirus phylogenetic tree based on the sequence of the L1 open reading frame. The numbers at the ends of each of the branches identify the Type; c-numbers refer to candidate types; all other abbreviations refer to animal papillomavirus types. The number at the inner semicircular symbols refer to the papillomavirus species and the outermost semicircular symbols identify papillomavirus genera. The tree was constructed using the MEGA5.1 program (33).

HPV-associated malignancies

Cervical cancer

The alpha genus comprises approximately 30 HPV types that infect the mucosa of the genital and oral tract as well as several benign cutaneous HPV types that are associated with the development of common skin warts. Based on their oncogenic potential, the mucosal HPV types are divided into two groups: low-risk HPVs (e.g. types 6 and 11) that are mainly associated with benign genital warts, and high-risk HPVs that are the etiological agents of cervical cancer (45). In a recent monograph, the International Agency for Research on Cancer as classified as carcinogenic to humans 12 different HR HPV types, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 (46). HPV16 and 18 are the most frequently found HPV types in cervical cancers worldwide, being detected in approximately 50% and 20% of the cases, respectively (47, 48).

The mucosal HPV types are sexually transmitted and in the female genital tract the area adjacent to the border of the endocervix and ectocervix, known as transformation zone or squamocolumnar junction, appears to be the preferential site for infection. It is believed that micro-traumas occurring during sexual intercourse strongly facilitate the viral infection. Approximately 80% of sexually-active women get a HPV infection during their life. In the majority of the cases, the HPV infections remain asymptomatic and are cleared by the immune system in a relatively short time (6–12 months). In a small number of cases, infection may persist, and after a period of latency, low and/or high-grade cervical intraepithelial neoplasia (CIN) develop, which may still regress or progress to an invasive cervical carcinoma (49). Impairment of the immune surveillance strongly facilitates viral persistence and subsequently HPV-induced carcinogenesis.

Penile Cancer

Cancer of the penis is common in developing countries (10 % of cancers in men), and is at least ten times higher than in the developed countries and mostly affects men aged between 50 and 70 years (50). Penile cancer has a multi-factorial etiology. The identified risk factors for the development of this cancer include poor hygiene, smoking, lack of circumcision, multiple sexual partners, and HPV infection (50, 51). The investigation of HPV involvement in penile

cancer development revealed a heterogeneous prevalence of high-risk HPV types. Thus not all penile cancer cases are HPV-related (prevalence of 46,9 %) (52). HPV detection in penile cancer revealed that HPV 16 is the most frequent HPV type associated to this cancer (60 %) (53). Histological analysis of the HPV-positive cases revealed that the basaloid and warty subtypes of penile carcinomas show the strongest association with the HPV (54-56). Penile cancer is generally treated through surgery; however this affects psychology and sexuality of the patient. Therefore, chemotherapy and radiotherapy are also implemented for penile carcinoma treatment (57, 58).

Anal cancer

Anal cancer represents 3 % of the colon and rectum malignancies and appears mainly at the age of 60 years (59). Rates of this type of cancer are continuously increasing (2 % per year) (60). This observation is at least partly attributed to the sexual behavior during the last decades. Anal cancer development is related to several risk factors including smoking, HIV-positivity, previous cancers (cervical, vulvar and vaginal cancers), sexual behavior, and HPV infection (61). As is for the cervical region, the anal region is very susceptible to HPV infection and the lesions form anal intraepithelial neoplasia (as observed in cervical cancer) that can progress to squamous cell carcinoma (SCC). HPV DNA was detected in 46 to 94 % of all anal cancers (62). In populations of HIV-positive men having sex with men, studies detected high-risk HPV types in approximately 90 % of the cases. According to the tumor extend, surgery and chemoradiotherapy are the most practiced therapies for anal cancer.

Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, it accounts for 3 to 4 % of all cancers (63, 64). Tobacco and alcohol use are traditionally associated with HNSCC development. However, thirty years ago, HPV was also suspected to play a role in the development of HNSCC (65). It is now well demonstrated that high-risk HPV types are also involved in a sub-set of head and neck cancers (HNC). Approximately, 25% of worldwide oropharyngeal carcinomas are linked to high-risk HPV infection, while the role of these viruses in HNC, such as oral cavity, larynx, and hypopharynx,

appears to be considerably less significant (66, 67). Among the high-risk HPV types, HPV16 is responsible for the majority (between 86-95%) of the HPV-positive oropharyngeal carcinomas (68). The higher prevalence of HPV16 in HNC in comparison to cervical cancer may be explained by intrinsic features of two distinct anatomical sites. In the oropharynx the immune system is much more active than the genital tract. Biological and epidemiological studies have shown that HPV16 is clearly more efficient in evading the host immune response than the other high-risk HPV types (69, 70). Thus, it is likely that HPV16 is more efficient than the other high-risk HPV types in establishing a persistent infection in the oropharynx, which is an essential step for the development of a malignant lesion.

Non-melanoma skin cancer

Non-melanoma skin cancer (NMSC) represents the most frequently diagnosed form of cancer among the Caucasian population, with continuing increase in incidence worldwide (71). The majority of NMSC are basal cell carcinomas (BCC), accounting for about 80 % of NMSC cases, and SCC account for the majority of the remaining NMSC (72, 73). It is well established that exposure to sunlight or ultraviolet A and B (UVA and UVB) can cause NMSC upon DNA damage (74). Fair skin, blond hair and blue eyes and immune status are also recognized as risk factors for NMSC (75-77). However, several studies indicate that HPV can be a co-factor favoring skin carcinogenesis, particularly beta HPV types. Genus beta comprises a large number of cutaneous HPV types that appears to be involved in the development of NMSC. Forty-three beta HPV types have been isolated and fully sequenced so far (78). Initial evidence for the possible association of beta HPV types with skin cancer came from the isolation of the first beta HPV types, HPV5 and HPV8, in the skin of cancer-prone patients suffering from a rare autosomal recessive genetic disorder called *Epidermodysplasia verruciformis* (EV) (30, 79). EV patients are highly susceptible to beta HPV type infections and develop disseminated pityriasis versicolor-like lesions and flat warts (80-82). These skin lesions arise early in life and in approximately 30–60 % of cases progress to multifocal SCC at sun-exposed regions. With the development of highly specific and sensitive detection methods, it became clear that beta HPV types are also abundantly present in the skin of normal individuals (83, 84). Investigation of the genetic background of EV patients identified a susceptibility locus within the chromosome 17. Mutations in EVER1 and EVER2 genes were therefore reported. In EV patients, a total of seven and five loss of function mutations were isolated in EVER1 and

EVER2 genes, respectively (85-87). These genes encode transmembrane proteins and also termed TMC6 (for EVER1) and TMC8 (for EVER2). They were recently reported to play a role in the regulation of zinc homeostasis that can have an effect on the HPV life cycle (88, 89). However, at present, it is still unclear whether EVER1 and EVER2 genes are involved in the immune response to HPV infection.

Epidemiological studies, using anti-HPV antibodies and/or viral DNA as markers of infection, have provided evidence that non-EV individual with history of skin SCC showed a higher positivity for beta HPV infections than controls (90-94). The fact that impairment of the immune system in organ-transplant recipients (OTRs) strongly increases the risk of NMSC further supports the role of an infectious agent in skin carcinogenesis (95-98). Accordingly, beta HPV infections were associated with an increased risk of NMSC in OTRs (99). Several data indicate that beta HPV types are involved at early stage of skin carcinogenesis. For instance, quantitative type-specific PCR revealed that higher viral genome copy number can be detected in pre-malignant lesions, actinic keratosis, than SCC (100, 101). In addition, far less than one HPV DNA copy per cell is present in NMSC biopsies (79). It is therefore likely that the presence of the viral genome may not be required in the later stages of carcinogenesis. This may imply a need to consider a new scenario for the role of HPV in NMSC pathogenesis, such as a "hit-and-run" mechanism. In this case, HPV viral DNA is required at the initiation phase facilitating the accumulation of DNA damage induced by UV-irradiation, but not for the maintenance of the transforming state.

HPV replicative cycle

Virus entry

Studies on the mucosal HPV types have shown that the first step in HPV infection is the interaction of the viral capsid with the cytoplasmic membrane of cells at basal layer of the epithelium. This event is mainly mediated by the major capsid protein L1 that interacts with to heparan sulfonated proteoglycan (HSPG) on the cell surface (102, 103). It is also possible that the viral particles bind another component of the cellular membrane. In fact, it has been proposed that $\alpha 6$ integrin may act as secondary cellular receptor for HPV particles (104). However, other studies showed that cells that do not express $\alpha 6$ integrin can be still infected by animal or human PV (102, 105). After binding of HPV16 particles to the cellular membrane, their internalization is mediated by a clathrin-dependent endocytic pathway (106). Additional findings indicate that other mucosal HPV types may use different endocytosis pathways (107). It is highly likely that the minor L2 capsid protein also plays a role in membrane binding and cellular internalization. In fact, anti-L2 antibodies against specific linear epitopes are able to block the internalization of L1/L2 virus-like particles in *in vitro* assays (108-110). Surviladze and colleagues have recently presented evidence for a novel mechanism of viral entry. They observed that after binding to the cell surface, HPV16 particles are released as a soluble complex with HSPG and growth factors. The latter proteins mediate the interaction of the soluble complex with their cognate receptors facilitating the internalization of the viral particles (111). Endocytosis *via* the clathrin is characterized by the fusion of the endosome with another type of endosome (e.g. lysosome) and the endocytosis via the caveoline system can lead to a direct fusion between the endosome and the endoplasmic reticulum. The length of virus entry may involve several molecular events that are responsible for the transfer of the virion from first contact of the cellular surface to specific receptors that mediate the entry (112). For instance, the keratinocytes extra-cellular matrix component Laminin 5 was found to be required for the entry of HPV11 (113). Following virus release from the endosome, L2 protein mediates the transfer to the cellular nucleus (114, 115). The N-terminus of L2 is cleaved within the endosomal compartment via the cellular protease, Furin, thus releasing an L2/genome complex into the cytosol (116). The L2/genome complex may then interact with Syntaxin 18, which ferries the complex to a perinuclear site (114, 116). L2 can be translocated in the nucleus

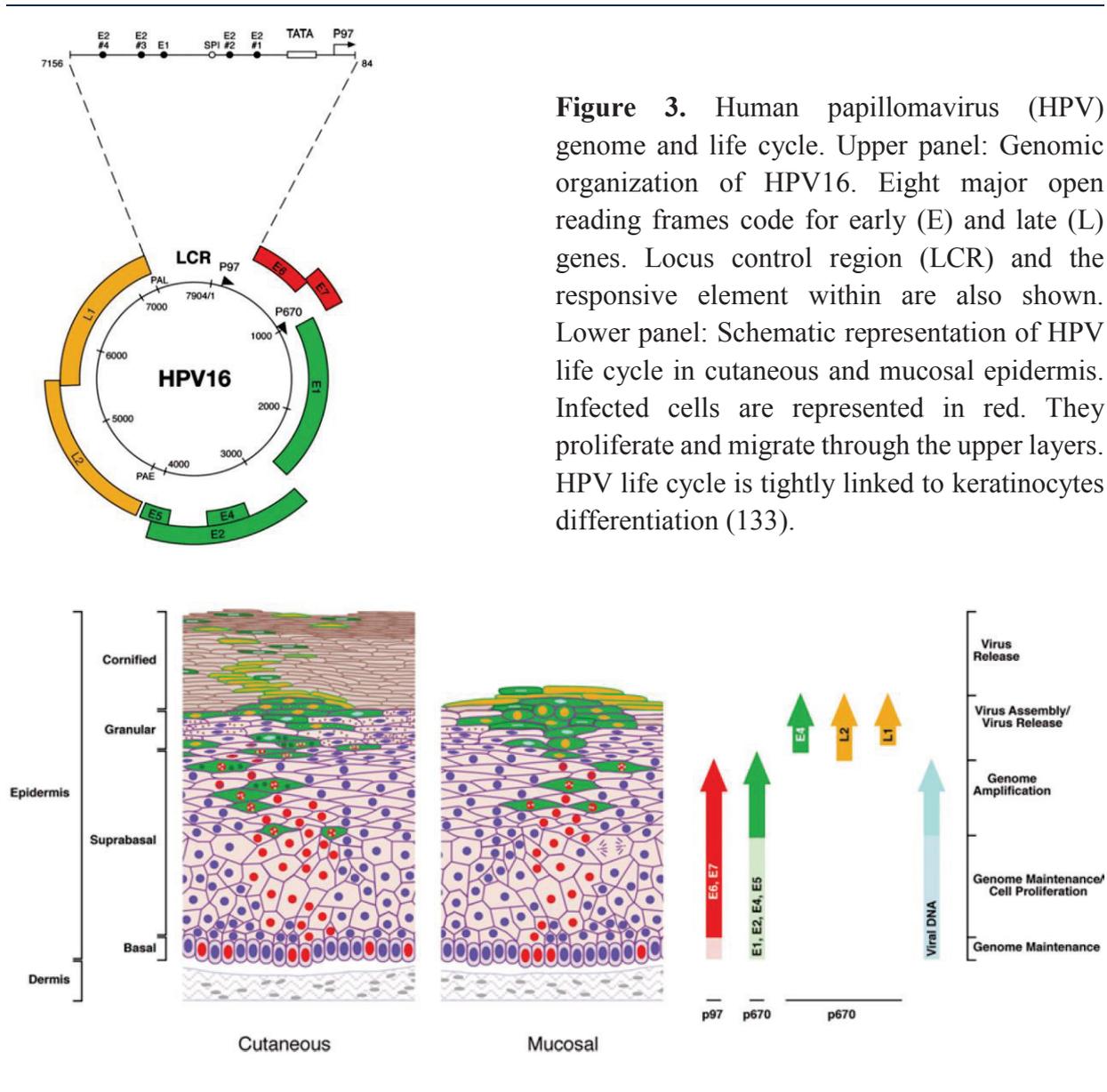
through its NLS (117). Following entry and uncoating, HPV genomes are replicated in the nucleus to about 100 episomal copies per cell.

Genome maintenance

Subsequent to its entry, the virus maintains a low-copy number of its genome in the host cell nucleus (118). The proteins E1 and E2 play important roles in the maintenance of the genome in episomal form and its segregation during cell division (118-120). E1 and E2 proteins bind to the origin of replication and recruit host polymerases necessary for replication. Their activity ensures the maintenance of the genome at a low copy number in the basal cells (121). During mitosis, viral genomes are partitioned to the daughter cells through the binding of E2 to viral DNA and to protein complexes on host cell chromosomes (122-125). This observation was further investigated using bovine PV (BPV) E2 protein. At specific steps of cell cycle, BPV E2 interacts with the DNA helicase ChlR1, suggesting the involvement of ChlR1 in the loading of the viral genome to the host cell chromosomes during DNA amplification (126, 127).

Genome amplification

The HPV genome is amplified in the suprabasal layer of the epithelium where keratinocytes undergo cell cycle arrest and differentiation. The E6 and E7 oncoproteins ensure a high proliferation rate of the cells by blocking cell cycle exit and inducing S-phase entry (128). Although host cell differentiation is necessary for the next steps of the viral life cycle, E7 forces cell cycle re-entry in differentiating cells by targeting a pRb family member, p130 (129, 130). Entry in differentiation process induces higher expression of the early proteins involved in replication (131). Therefore, this event induces a high production of viral genome that can be ranged at thousands per cells (128, 132).



Virion synthesis

The assembly and release of the virions are dependent on the expression of the late proteins L1 and L2 and loss of E6 and E7 expression in the upper layer of the epithelium (Figure 3) (134-136). L1 and L2 proteins form capsomers in the cytoplasm and translocate into the nucleus thanks to nuclear localization signals using karyopherins (137). Virus assembly occurs in promyelocytic leukemia protein (PML) nuclear bodies, also referred as nuclear domains 10 (ND10). All virus components are recruited to the ND10 via an important role played by the capsid protein L2 (138). The viral genome is recruited by E2 protein to the ND10 assembly sites (139). Following the packaging, virions undergo the last phase of their life cycle by releasing from the cells and escaping from the cornified envelop (140). During the virus life cycle, cells are forced to proliferate, accumulating DNA damage and deregulating their homeostasis. HPV persistence can lead to malignant lesions and even cancers for some cases.

Human papillomavirus E6 and E7 roles in carcinogenesis

One of the first pieces of evidence for the oncogenic role of E6 and E7 was provided by the analysis of cervical cancer-derived cell lines. In many of these cells, HPV genome was found integrated into the host genome. This integration generally leads to disruption of the majority of viral genes with only preservation of the E6 and E7 genes that are efficiently expressed (141). Silencing the expression of E6 and E7 genes in cervical cancer cell lines results in rapid death (142). E6 and E7 are small proteins of approximately 150 and 100 amino acids, respectively. They have the capacity to alter important functions of the host cells resulting in their immortalization. E6 and E7 have several strategies to evade immunesurveillance, to alter cell cycle control, and to inhibit apoptosis. In the normal virus life cycle, these functions allow viral persistence and replication but, as side effect, cause the accumulation of DNA damage and subsequent malignant transformation. Both viral oncoproteins exert these activities by directly interacting with a vast number of cellular proteins. Several E6 and E7 functions are shared with many HPV types, while some are exclusive features of E6 and E7 belonging to the high-risk HPV types. Among all HPV types, the high-risk HPV16 is the most investigated and therefore many of its E6 and E7 functions have been fully characterized. In the contrary, only few studies have investigated the E6 and E7 properties from beta HPV types (143).

Major transforming activities of E6 and E7 from alpha HPV types

High-risk HPV E6 properties

The best characterized activities of HPV16 E6 and E7 are their ability to target tumor suppressors, p53 and pRb respectively. The functions of E7 in the host cell cause an abnormal increase in DNA replication resulting in cellular stress. In this situation the host cell would activate apoptosis through p53; however, E6 protein from high-risk HPV types is able to inhibit the p53-dependent programmed cell death. To counteract the cellular response to the E7-mediated unscheduled proliferation, E6 binds p53 and causes its proteasome-dependent degradation (143).

The interaction between E6 and p53 was the first activity described for high-risk HPV E6 proteins (144). In a normal situation, cells maintain very low levels of p53 through the ubiquitin ligase mdm2 that is able to directly interact with p53 and to promote degradation via the proteasome pathway (145-147). Upon cellular stresses, several events lead to the loss of mdm2/p53 interaction, with strong stabilization of p53, which in turn promotes cellular arrest or apoptosis (148). In HPV16-infected cells, The E6 oncoprotein mediates the interaction of p53 with another ubiquitin ligase enzyme, E6 associated protein (E6AP), promoting the degradation of the tumour suppressor (149, 150). Thus HPV-positive cells display a relatively low expression of p53-regulated genes upon genotoxic stress, facilitating the accumulation of DNA damage and, ultimately, cellular transformation (151, 152).

In addition, E6 has developed other mechanisms to inhibit p53 transcriptional activity. It has been shown that E6 binds the transcriptional coactivators p300/CBP (CREB binding protein) that play a positive role in p53-regulated transcription *via* p53 acetylation (153-155). E6 protein inhibits another p53 coactivator, hADA3, that is part of acetyltransferases complex (156). This protein is degraded through an E6/E6AP-dependent process (157). Decreased levels of hADA3 correlate with the inhibition of p14ARF/p53 pathway and subsequent cellular immortalization of mammalian epithelial cells (158). G protein pathway suppressor 2 (GPS2) is another E6-targeted transcription coactivator, that is known to inhibit RAS- and MAPK-mediated signaling (159). GPS2 protein is degraded upon interaction with E6 (159). In addition to affecting p53-induced apoptosis E6 has also an effect on the mitochondrial- and death receptors-related apoptosis. E6 was shown to bind, together with E6AP, the protein Bak leading to its degradation (160). Bax mRNA levels are also affected by E6, downregulating its protein levels (161). Moreover, E6 inhibits tumor necrosis factor (TNF) signaling. It binds to the TNF-receptor 1 and blocks the interaction with Fas associated death domain (FADD) (162-164).

High-risk HPV E7 properties

E7 proteins from high-risk HPV types can interact with pRb, p107, and p130 proteins, also termed pocket proteins (165, 166). The protein E7 is able to induce proteasome-dependent pRb degradation. During the G1/G0 phase of uninfected cells, the pocket proteins interact with the transactivating domain of E2F transcription factors (167). Upon exposure of the cells to proliferative stimuli, pRb is phosphorylated by the G1 cyclin-dependent kinase (CDK) complexes and loses the ability to interact with E2F. In turn, free E2F drives the transcription of key genes that encode proteins involved in cell cycle progression (167). In HPV-infected cells, E7, via degradation of pRb, induces constitutive activation of the G1/S transition, and induces the cellular proliferation independent of external proliferative stimuli (168-170). HPV16 E7 was reported to bind the ubiquitin ligase cullin 2, and this interaction is important for cellular transformation and cell cycle checkpoints deregulation. These observations suggest the contribution of cullin 2 in E7-mediated pRb destabilization (171).

Transition from G1/S to the S phase is also achieved by the induction of cyclin A/CDK2 and as reported for E6, E7 also has redundant mechanisms for deregulation of the cell cycle control proteins (172-174). Indeed, E7 is able to directly associate with two CDK2 inhibitors, p21Cip1 and p27Kip1, with consequent block of their functions (175, 176). Beyond the role as regulator of cell cycle, it has been shown that pRb is involved in other cellular events, e.g. differentiation. Therefore, the E7-mediated pRb degradation also results in inhibition of other cellular functions.

E7 from HPV16 has also been reported to deregulate the transcription of co-activators p300, CBP, and p/CAF (p300/CBP-associated protein) (177-179). These proteins are involved in the activation of E2F-mediated gene transcription (180). Similarly, E7 binds to histone acetyl transferases (HAT) and increases the levels of histone H3 acetylation within E2F-target gene promoters correlating with active transcription (181). In addition to binding pRb, E7 acts also on the histone deacetylases (HDAC) which remove acetyl groups from histones resulting in the formation of heterochromatin. High-risk HPV E7 binds to HDAC through the intermediate binding of Mi2 β , a chromatin remodeling protein (182). The C-terminal domain of E7 plays an essential role in this interaction. Sequestration of HDAC by E7 results in the induction of gene expression following chromatin remodeling due to histone acetylation. Blocking the interaction of HDAC and pRb with E7 results, respectively, in the destabilization of viral genome maintenance and cell cycle progression during differentiation (182-185). This demonstrates the

importance of pRb and HDAC binding and inactivation by E7 during the process of keratinocytes immortalization.

Deregulation of NF- κ B pathway

Nuclear Factor- κ B (NF- κ B) constitutes a family of transcription factors: NF- κ B1 (p50 and its precursor p105), NF- κ B2 (p52 and its precursor p100), RelA (p65), RelB and cRel (Rel). These proteins can form heterodimer or homodimers. The NF- κ B protein family shares a highly conserved N-terminal region, known as the Rel homology domain (RHD), which is involved in dimerization, DNA binding sequence, interaction with the inhibitory I κ B proteins, and contains a nuclear localization sequence (NLS). In a latent state, NF- κ B dimers are mainly localized in the cytoplasm, sequestered by binding the inhibitory subunits I κ B. As NF- κ B, I κ B proteins form a family containing several members. In mammals, it includes I κ B α , I κ B β , I κ B γ , I κ B ϵ , Bcl-3, and the precursor Rel-proteins, p100, and p105. Activating stimuli mediate the 26S proteasome-dependent degradation of I κ B, following its phosphorylation by I κ B kinases (IKK) at two conserved serine residues (S32 and S36) in its N-terminal regulatory domain. Then, NF- κ B dimer is free to translocate to the nucleus, it binds specific κ B sites and regulates the expression of a target gene (186).

As shown in figure 4, the canonical or classical pathway is characterized by the NF- κ B dimer p50 and p65. Its activation is regulated by the IKK complex which is composed of the regulatory subunit IKK γ (NEMO) and the catalytic subunits IKK α and β . Following stimulation by different agents such as TNF- α or IL-1, viruses, genotoxic agents, and ionizing radiation, this pathway leads to the expression of several genes crucial for the activation of innate immunity and inflammation as well as modulation of apoptosis. Two other activating pathways included in the canonical one are not fully elucidated. The first is induced by DNA damage which activates casein kinase 2 (CK2). The second acts in response to phorbol esters, lipopolysaccharide (LPS), and cytokines stimuli, and involves the activation of IKK ϵ and TANK-binding kinase (TBK). The other major NF- κ B signaling is known as the alternative or non-canonical pathway. It is activated by several stimuli such as B-cell-activating factor, LT α/β , or CD40L, leading to the NF- κ B-inducing kinase (NIK)-dependent activation of the homodimer IKK α . RelB and p100 form the NF- κ B dimer which is activated by the degradation

of the C-terminal region of p100 after phosphorylation by IKK α . This pathway is required for B cell maturation and survival, and formation of secondary lymphoid organs (187-189).

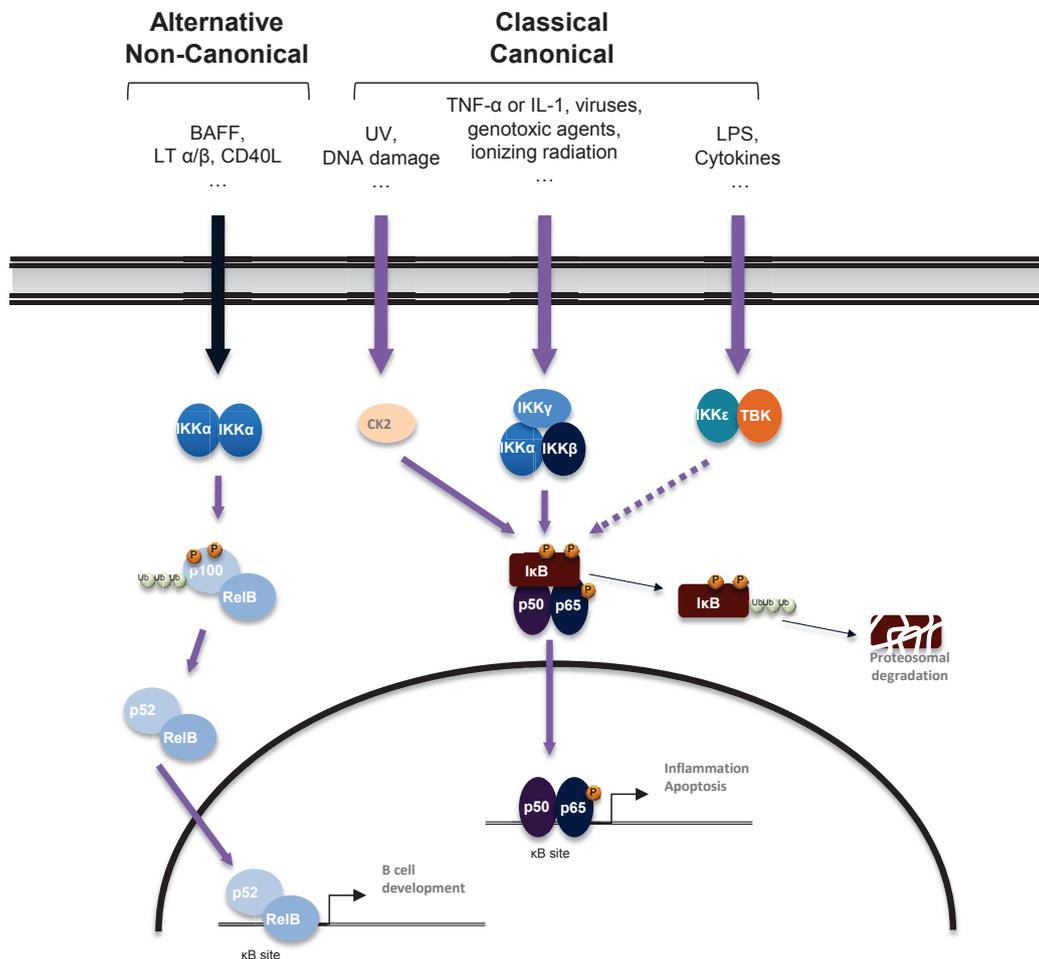


Figure 4. Schematic representation of NF- κ B signaling.

NF- κ B canonical pathway is characterized by the p50/p65 NF- κ B subunits which are inhibited by I κ B. The phosphorylation of I κ B by CK2, IKK ϵ or IKK complex leads to its degradation. This pathway targets genes implicated in inflammation and apoptosis. The non-canonical NF- κ B pathway involves the p100/RelB complex which is targeted by the IKK α homodimer. Phosphorylation of p100 causes its processing to the active form p52. The activation of this pathway is mainly responsible for the expression of genes involved in B cell development

NF- κ B signaling is found activated in many cancers and targeted by oncogenic viruses including EBV, HPV, and HTLV (190-193). These viruses have developed several strategies to take advantage of the ability of NF- κ B to induce for instance anti-apoptotic and growth-promoting genes. In correlation with the presence of HPV, the NF- κ B pathway is found

overexpressed in penile and cervical cancer cases (194-196). In laryngeal SCC cells infected with HPV, a positive correlation has been reported between the level of HPV16 E7 and the nuclear localization of the p65 subunit of NF- κ B (197). A study carried out on oral tissue biopsies, showed that in high-risk HPV positive tumors the positive transcriptional regulatory complex p65/p50 is more abundant than the homodimer complex p50/p50, which is often detected in HPV-negative cancers (197, 198). Interestingly, in airway epithelium, it was observed that HPV16 E6-expressing cells have an increased transcription of the NF- κ B-regulated anti-apoptotic gene cIAP-2 (193). E6 induces this event via the induction of the nuclear binding activity of p52 and it requires the E6 PDZ binding motif (193). This function of E6 is important to protect HPV infected cells from TNF-induced apoptosis. Furthermore, expression of E6 and E7 in cervical keratinocytes leads to pro-proliferation-specific activation of NF- κ B (199). In contrast with these observations, some studies have reported that HPV16 oncoproteins can reduce NF- κ B signaling. HPV16 E6 was shown to attenuate p65 transcriptional activity and E7 to target IKK complex and reduces NF- κ B activity (200, 201). They inhibit interferon signals and their target genes. These effects of HPV oncoproteins could be important to counteract the immune sensing of the infected cells, and these functions could be in line with the evasion of the immune system allowing the virus to persist in the host cells.

Deregulation of hTERT

Telomeres' length is shortened after each cell cycle causing, at the Hayflick limit, genomic instability and cellular senescence. A normal cell undergoes apoptosis before reaching this limit, but tumor cells bypass this limit and proliferate continuously (202). Different mechanisms of maintaining telomere length have been described. E6 was shown to activate the catalytic subunit human telomerase reverse transcriptase (hTERT) of the ribonucleoprotein complex responsible for the synthesis of telomere repetitions (203-205). Several studies have shown that high-risk HPV E6 can activate telomerase activity by independent mechanisms. For instance, E6 induces hTERT transcription *via* activation of c-Myc which binds on the promoter of hTERT and positively regulates its transcription (206, 207). In addition, E6, *via* the interaction with E6AP, promotes the degradation of a negative regulator of hTERT transcription, nuclear transcription factor X-box binding 1-9 (NFX1-91) (208, 209). Another splice form of NFX1,

NFX1-123, is not affected by E6 and E6AP. This form of NFX1 acts positively on hTERT levels by increasing its mRNA stability (210, 211).

Deregulation of PDZ proteins

Another well characterized property of E6 from high-risk HPV types is the ability to target several members of the membrane-associated guanylate kinase (MAGUK) family that are involved in the regulation of cell-cell contact *via* the tight junction and cell polarity (212-214). They contain various protein/protein interaction domains, including PDZ motifs. The term PDZ refers to the first letters of the three first proteins in which a consensus domain of approximately 90 amino acids was identified. These proteins are: PSD (PostSynaptic Density), DLG (Drosophila disc large protein), and ZO1 (Zonula Occludens 1) (166).

The high-risk HPV16 E6 oncoproteins have a four amino acid PDZ-binding motif at the C-terminus that mediates the interaction with the MAGUK family members. The PDZ-binding motif of E6 protein is important for its transforming activity. Mutation of this motif results in the inhibition of cellular growth and viral DNA replication (215-218). Upon interaction, E6 induces the degradation of PDZ proteins leading to the loss of control of pathways that regulate cellular adherence and polarity (214, 219). Multiple PDZ proteins have been reported as E6 substrates including hDlg1, MAGI-1, and hScrib (214, 220, 221). These events could increase the metastatic dissemination of the HPV-infected cells.

Major transforming activities of E6 and E7 from beta HPV types

As mentioned above, the molecular mechanisms and role of beta HPV types in skin carcinogenesis are still not sufficiently investigated. However, several initial studies provide evidence that E6 and E7 from specific beta HPV types can deregulate important cellular functions and lead to cellular immortalization. In particular, it has been shown that E6 and E7 from two beta HPV types, 38 and 49, are able to efficiently immortalize primary human keratinocytes (222-225). These observations support the putative etiological role of beta HPV types in NMSC development.

Animal models

The implication of cutaneous beta HPV in skin cancer was investigated in animal model through the expression of several early genes in the skin using tissue-specific promoters such as keratin 10 (K10) or keratin 14 (K14) promoters. Transgenic mice expressing HPV8 E2, E6, and E7 proteins in their skin display a higher risk to developing NMSC (226-229). Another study has shown that the expression of HPV20 E6 and E7 proteins in mice skin induces cutaneous cells hyperproliferation and inhibition of cell differentiation upon UVB-irradiation. Similar observations were made with the expression of HPV38 E6 and E7. The exposure of transgenic mice to UVB with a skin-specific expression of HPV38 E6 and E7 leads to hyperproliferation and p21 transcription inhibition (230, 231). The exposure of the same transgenic mice to chemical carcinogens, dimethylbenzanthracen (DMBA) and trimethylamin (TPA), enhanced the development of cutaneous epidermoid carcinomas compared to the control mice (230, 232). Another study has shown that transgenic mice expressing HPV8 E2 or E6 protein developed skin tumors spontaneously or upon UV-irradiation (228, 233).

All together, these findings demonstrate the carcinogenic capacity of some beta HPV early proteins in *in vivo* models. This risk of tumor development in the presence of the cited viral proteins appears to act in synergism with other carcinogens.

Deregulation of p53 pathway

In contrast to E6 from mucosal alpha HPV types, those from cutaneous beta HPV types did not show the ability to degrade p53. However, a recent study has pointed out the destabilization of p53 protein levels in human keratinocytes expressing HPV49 E6 and E7, in line with their ability to immortalize human primary keratinocytes (224). The protein E6 from this beta HPV type appeared to bind p53 together with E6AP. The formation of this complex leads to p53 proteolysis that is not as efficient as with HPV16 E6 suggesting possible differences in this process between the two HPV types (224). HPV49 E6 is, so far, the only beta HPV protein shown to act directly on p53 protein stability.

Studies on beta HPV types also led to the characterization of a novel mechanism involved in the regulation of intracellular levels of $\Delta Np73\alpha$, an antagonist of the p53/p73-regulated pathways (234, 235). HPV38 E6 and E7 promote the accumulation of a specific form of p53 that positively regulates the transcription of $\Delta Np73\alpha$ (234). In addition, HPV38 acts on a post-translational level increasing $\Delta Np73\alpha$ half-life (235). HPV38 E6 and E7 expression in primary human keratinocytes promotes the translocation of I κ B-kinase- β (IKK β) in the nucleus, which in turn associates with and phosphorylates $\Delta Np73\alpha$ at serine 422, leading to its stabilization and repression of several p53-regulated genes. Inhibition of IKK β results in a rapid degradation of $\Delta Np73\alpha$ and a rescue of p53 function (235).

In addition, E6 from HPV23 was shown to block p53 activation by preventing its phosphorylation. HPV23 E6 binds to HIPK2 and hampers its interaction with p53, necessary for its phosphorylation at Ser46 (236). This activity of HPV23 E6 in human keratinocytes is important for the survival of the cells upon UV-exposure (236). Cutaneous HPV E6 is also able to inhibit the UV-induced apoptosis in the presence of high levels of active p53 protein (237). Apart of acting directly on p53 function, E6 can inhibit apoptosis independently of p53. It has been shown that E6 interacts with the pro-apoptotic protein Bak which is involved in mitochondria-mediated induction of apoptosis for instance upon UV-exposure (238). In cells irradiated with UV and expressing E6 from HPV5, and other cutaneous HPV types of different genera (HPV10 and 77), Bak-mediated apoptosis is inhibited (238). This protein was shown to be degraded through ubiquitin-ligase interaction with E6 from cutaneous HPV5, 8, 10 and 77 (238). These features of beta HPV E6 proteins to block UV-mediated apoptosis reinforce the hypothesis of a possible synergism between HPV and UV-induced DNA damage during skin cancer development. In line with the inhibition of UV-induced apoptosis, E6 was moreover

shown to affect DNA damage repair pathways. This results in the accumulation of DNA damage and double strand breaks leading to chromosomal instability. UV-exposure of the cells induces the formation of cyclobutane pyrimidine dimers that are the predominant DNA lesions in human skin cells. HPV5 E6 is able to affect the repair of this DNA damage when expressed in epidermal cells (239). The XRCC1 protein, implicated in single strand DNA breaks repair, has been also reported to be targeted and inhibited by HPV8 E6 (240).

Deregulation of pRb pathway

The knowledge on cutaneous beta HPV E7 proteins is extremely limited compared to those from mucosal alpha HPV types. The most relevant finding for cutaneous beta HPV E7 to mention is the characterization of some biological properties of HPV38 E7. It has been reported that this protein is able to interact with pRb protein, as efficiently as seen for HPV16 E7, in human keratinocytes (222, 224). This function of HPV38 E7 deregulates cell cycle check points in G1 and S phases, contributing to the transformation of the host cell (222). The observed effect on pRb by HPV38 E7 does not occur in presence of beta HPV20 E7 proteins (224). A comparative study of the effects of E7 proteins from several beta HPV types highlighted the efficiency of HPV38 E7, and HPV49 E7, in deregulating the pRb pathway. The expression of HPV49 E7 in human keratinocytes appeared to inhibit the inactivation of E2F by pRb and enhanced the expression of cell cycle regulators such as cMyc, cyclin A, and Cdc2 (224).

Deregulation of NF- κ B pathway

As exposed above, despite the important place of NF- κ B pathway in cellular homeostasis through its involvement in several functions such as inflammation and cellular growth balance, very few studies have investigated its deregulation upon the expression of beta HPV proteins. A recent study has investigated the effect of E7 proteins from several cutaneous HPV types in U2OS cells, including the E7 proteins from beta HPV20, 37, 38, 92, 93, and 96 (241). In these cells, the investigators have reported the inhibition of NF- κ B activity in presence of E7 proteins that are able to bind IKK α but not IKK β (241). This finding is in line with what has been shown for HPV16 E7 in the same cellular model (200). Additional experimental analysis will be

required to understand the effect of NF- κ B deregulation. In the present thesis we will explore the status of NF- κ B pathway in human keratinocytes expressing HPV38 E6 and E7.

Deregulation of hTERT

It is now well established that although HPV38 E6 and E7 fail to degrade p53, they possess efficient mechanisms to inhibit cellular cell cycle control and apoptosis induction (234). It has also been shown that human primary keratinocytes expressing HPV38 E6 and E7 gradually erode their telomeres (223). The shortening in telomere length correlates with genomic instability and chromosomal rearrangements that can result in the transformation of some cellular clones. The same study has shown that the transcriptional activation of hTERT expression can be at least partially inhibited by the knockdown of Δ Np73 α protein synthesis (223). This observation suggests the involvement of Δ Np73 α in the overexpression of hTERT in presence of HPV38 E6 and E7. Another study has investigated the status of hTERT expression in keratinocytes by comparing the effects of the expression E6 and E7 from different beta HPV types. E6 and E7 from HPV38 and HPV49 were the most significantly inducing hTERT expression in human keratinocytes (224). It was also reported that E6AP plays an important role in the beta HPV-mediated hTERT activation (242). E6 proteins from different beta HPV type interact with E6AP and NFX1-91 and induce telomerase activity (242).

Deregulation of Notch pathway

Notch was described to play an important role in keratinocyte differentiation and cell cycle arrest processes (243). In human keratinocytes, Notch has been reported to act as a tumor suppressor (244). Several studies investigated the status of Notch signaling in presence of beta HPV proteins. It has been shown that E6 from several beta HPV types are able to inhibit Notch signaling (245). E6 proteins from HPV8 and as well as cutaneous HPV1 associate mastermind-like 1 (MAML1), a NOTCH coactivator, and repress the expression of Notch target genes (246, 247). These findings highlight the potential important role of Notch pathway inhibition during HPV infection and the need of further characterization of the molecular mechanism.

Aim of the thesis

Viral infections trigger numerous host cell defenses. A well-known cellular protection against viral infection and spread is the suicide of the infected cells. To complete the viral life cycle, several viruses adopt complex strategies to evade apoptotic cell death. The molecular mechanism of high-risk HPV E6 and E7-mediated targeting of important cellular pathways such as p53 signaling is well described. However, knowledge on beta HPV E6 and E7 functions toward cellular transformation is still poor. In this thesis we have studied HPV38 E6 and E7 activities in deregulating p53 and NF- κ B pathways.

First study:

It has been shown that p53 functions are inhibited in HPV38 E6 and E7-expressing keratinocytes through the accumulation of Δ Np73 α . In this study we have investigated the individual contribution of E6 and E7 in IKK β -nuclear accumulation and Δ Np73 α stabilization. We have also further characterized the cooperation between IKK β and Δ Np73 α to achieve the transcriptional inhibition of p53-regulated genes.

Second study:

In contrast to high-risk HPV types that are known to deregulate NF- κ B, we have limited knowledge about the influence of beta HPV early proteins expression on this pathway. In this study we have investigated the effects of HPV38 E6 and E7 proteins on the activity of NF- κ B signaling. We have also evaluated the role of NF- κ B in E6 and E7-mediated apoptosis inhibition. Finally we asked which of E6 and E7 is responsible of NF- κ B activation and through what potential mechanism.

Chapter II: Results 1

The E7 oncoprotein from beta human papillomavirus type 38 induces the formation of an inhibitory complex for a subset of p53-regulated promoters

**The E7 oncoprotein from beta human papillomavirus type 38 induces the formation of an
inhibitory complex for a subset of p53-regulated promoters**

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Abstract

1 Our previous studies on cutaneous human papillomavirus (HPV) type 38 E6 and E7 oncoproteins
2 highlighted a novel activity of I κ B kinase beta (IKK β) in the nucleus of human keratinocytes, where it
3 phosphorylates and stabilizes Δ Np73 α , an antagonist of p53/p73 functions. Here, we further characterize
4 the role of the IKK β nuclear form. We show that IKK β nuclear translocation and Δ Np73 α accumulation
5 are mediated mainly by HPV38 E7 oncoprotein. ChIP/Re-ChIP experiments showed that Δ Np73 α and
6 IKK β are part, together with two epigenetic enzymes DNA methyltransferase 1 (DNMT1) and enhancer
7 of zeste homolog 2 (EZH2), of a transcriptional regulatory complex that inhibits the expression of some
8 p53-regulated genes, such as *PIG3*. Recruitment to the *PIG3* promoter of EZH2 and DNMT1 resulted in
9 tri-methylation of histone 3 on lysine 27 and in DNA methylation, respectively, both events associated
10 with gene expression silencing. Decreases in the intracellular levels of HPV38 E7 or Δ Np73 α strongly
11 affected the recruitment of the inhibitory transcriptional complex to the *PIG3* promoter, with consequent
12 restoration of p53-regulated gene expression. Finally, the Δ Np73 α /IKK β /DNMT1/EZH2 complex
13 appears to bind a subset of p53-regulated promoters. In fact, the complex is efficiently recruited to
14 several promoters of genes encoding proteins involved in DNA repair and apoptosis, whereas it does not
15 influence the expression of the pro-survival factor Survivin. In summary, our data show that HPV38 via
16 E7 protein promotes the formation of a multiprotein complex that negatively regulates the expression of
17 several p53-regulated genes.

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23 Introduction

24 A sub-group of cutaneous human papillomaviruses (HPV) that belongs to the genus beta of the HPV
25 phylogenetic tree are suspected to be involved, together with ultraviolet (UV) radiation, in the
26 development of non-melanoma skin cancer (1, 2). Beta HPV types were originally isolated in patients
27 suffering from a rare autosomal recessive cancer-prone genetic disorder, epidermodysplasia
28 verruciformis (EV), and are consistently detected in non-melanoma skin cancer (NMSC) from EV
29 patients and immunocompromised and normal individuals (2). Many independent studies have
30 demonstrated that the E6 and E7 proteins of several beta HPV types display transforming activities in *in*
31 *vitro* and *in vivo* experimental models (3-12). We previously showed that E6 and E7 of beta HPV type
32 38 are able to efficiently immortalize primary human keratinocytes (4, 6). In addition, constitutive
33 expression of HPV38 E6 and E7 in the skin of Transgenic (Tg) mice increases their susceptibility to
34 chemical or UV-induced skin carcinogenesis (13).

35 The transforming activity of HPV38 is partly mediated by the accumulation of $\Delta\text{Np73}\alpha$ (3, 6, 14, 15), an
36 antagonist of p53/p73 functions involved in growth suppression and/or apoptosis (16-18).
37 $\Delta\text{Np73}\alpha$ cooperates with the oncogenic Ras^{V12} in inducing transformation of primary mouse embryonic
38 fibroblasts (MEF), which acquire the property of inducing tumors upon injection into nude mice (19,
39 20). In addition, MEF from $\Delta\text{Np73}\alpha$ -/- mice expressing the adenoviral E1A oncoprotein together with
40 Ras^{V12} are impaired in their ability to induce tumor formation when injected into athymic mice (21).
41 Elevated $\Delta\text{Np73}\alpha$ levels were found in some human cancers with poor prognosis (17, 22-24).

42 Similarly to HPV38, other viruses such as cytomegalovirus and Epstein-Barr virus (EBV) were shown to
43 be able to induce accumulation of $\Delta\text{Np73}\alpha$ by different mechanisms (3, 14, 25, 26). We previously
44 showed that expression of HPV38 E6 and E7 in keratinocytes or of EBV LMP-1 in B cells promotes
45 $\Delta\text{Np73}\alpha$ transcriptional activation (3, 14). In contrast, cytomegalovirus does not alter $\Delta\text{Np73}\alpha$ mRNA

46 levels, but promotes a rapid increase in $\Delta\text{Np73}\alpha$ protein levels, most likely influencing its stability (26).
47 We recently showed that HPV38, similarly to cytomegalovirus, is also able to increase $\Delta\text{Np73}\alpha$ protein
48 levels independent of its effect on gene expression (14). This event is mediated by the translocation of
49 I κ B kinase β (IKK β) into the nucleus, where it binds and phosphorylates $\Delta\text{Np73}\alpha$ at serine 422 (S422).
50 Phosphorylation of S422 significantly increases $\Delta\text{Np73}\alpha$ half-life, resulting in transcriptional inhibition
51 of p53-regulated genes, such as *p21*, *PIG3*, *Fas*, and *Mdm2*. In this study, we evaluated the contribution
52 of individual HPV38 oncoproteins in IKK β nuclear translocation and $\Delta\text{Np73}\alpha$ stabilization, and we
53 further characterized the role of IKK β and $\Delta\text{Np73}\alpha$ in transcriptional regulation of p53-regulated genes.

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69 **Materials and Methods**

70 **Plasmid constructs**

71 The genes of interest were expressed in the following expression vectors: pBabe (27), pLXSN
 72 (Clontech, BD, Le Pont Claix, France), pRetroSuper (Screeninc, Amsterdam, The Netherlands) and
 73 pcDNA3.1 (Invitrogen). The constructs pRetroSuper-HPV38 E6E7, pBabe-puro-Flag-DN-IKK β
 74 (dominant-negative kinase-dead IKK β), pBabe-puro- Δ N-I κ B α (lacking the first 36 N-terminal amino
 75 acids), pLXSN-HPV38 E6E7, pRetroSuper-HPV38 E6E7, pcDNA3-HA- Δ Np73 α , pcDNA3-Flag-IKK β ,
 76 and pBabe-hygro-CRE were previously described (6, 14). The construct pBabe-puro-HPV38
 77 E6E7^{LoxP/LoxP} was generated by cloning HPV38 E6E7 open reading frames into the pBabe-LoxP-puro
 78 vector. p53RE-mutated PIG3 promoter was generated with the QuikChange Lightning Site-Directed
 79 Mutagenesis kit (Qiagen) using pGL3-wild-type PIG3 promoter vector as template and the following
 80 oligonucleotides: Sense 5'-GCAGCACCCAGATTACCCACCTATACTCAAGATGGGCGGG-3' and
 81 antisense 5'-CCCGCCCATCTTGAGTATAGGTGGGTAATCTGGGTGCTGC-3'.

82

83 **Cell culture and treatment**

84 Human foreskin keratinocytes (HFK) were grown together with NIH 3T3 feeder layers in FAD medium
 85 containing 3 parts Ham's F12, 1 part DMEM, 5% fetal calf serum, insulin (5 μ g/ml), epidermal growth
 86 factor (10 ng/ml), cholera toxin (8.4 ng/ml), adenine (24 μ g/ml) and hydrocortisone (0.4 μ g/ml). Feeder
 87 layers were prepared by mitomycin C-induced cell cycle arrest of NIH 3T3 cells.
 88 HFK stably expressing HPV38 E6 or E7 were generated by retroviral transduction. Cells transduced
 89 with the empty retroviral vector (pLXSN) were used as negative control. Due to the limited life span of
 90 HFK expressing HPV38 E6 or E7, the experiments shown in Figure 4B were performed with cells
 91 expressing HTERT together with each of the viral proteins. For DNA methylation analysis, HFK

92 expressing HPV38 E7 and E6 genes (38E6E7 HFK) were treated with 10 μ M 5-aza-2'-deoxycytidine (5-
 93 Aza; Sigma-Aldrich) or DMSO for 4 days with drug replacement every 24 h. At the end of the
 94 treatment, cells were collected for DNA methylation and RT-qPCR analysis. For transient transfection,
 95 38E6E7 HFK (1×10^5 cells/well) were seeded in a 24-well plate and transfected using JetPrime reagent
 96 (Polyplus) according to the manufacturer's protocol.

97

98 **Colony formation assay**

99 For colony formation assays, the different types of cells were seeded at different dilutions (10^4 , 10^3 and
 100 10^2) and cultured for 7 days. Cells were then stained with crystal violet and the average number of cells
 101 per colony was counted.

102

103 **Gene expression silencing**

104 Down-regulation of Δ Np73 α was achieved using antisense oligonucleotide (AntiSense) (Table 1) as
 105 previously described (3, 14). EZH2 and DNMT1 silencing was performed using specific Stealth siRNA
 106 and Scrambled siRNA as control designed using BLOCK-iT RNAi designer (Invitrogen). The target
 107 sequence for EZH2 is 5'-AGUGGUGCUGAAGCCUCAUGUUUA-3' and for DNMT1 is 5'-
 108 UUGGAGAACGGUGCUCAUGCUUACA-3'. The cells were transfected with 250 nM siRNA using
 109 Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's protocol. The cells
 110 were collected 72 h after transfection or stained with crystal violet after 7 days.

111 Silencing of HPV38 E6E7 gene expression was obtained using either (i) the pRetroSuper construct
 112 (pRS), expressing small hairpin RNAs (shRNAs) for the polycistronic HPV38 E6 and E7 mRNA (pRS
 113 38E6/E7) (3) or (ii) the Cre/*LoxP* system. For the latter approach, HFK transduced with pBabe-puro-
 114 HPV38 E6E7^{*LoxP/LoxP*} retroviruses were selected for 5 days with puromycin and cultured for several

115 weeks until immortalization. Subsequently, cells were transduced with pBabe-hygro or pBabe-hygro-
116 CRE retrovirus and cultured in the presence of hygromycin.

117

118 **RT-PCR and quantitative PCR**

119 Total RNA was extracted using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) and
120 quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). For RT-
121 PCR, the obtained RNA was reverse transcribed to cDNA with the RevertAid H Minus M-MuLV
122 Reverse Transcriptase kit (MBI Fermentas GmbH, St. Leon-Rot, Germany) according to the
123 manufacturer's instructions. Real-time qPCR was performed using the Mesa Green qPCR MasterMix
124 Plus for SYBR Assay (Eurogentec, San Diego, USA) with the primers listed in Table 1.

125

126 **Immunofluorescence staining**

127 HFK were grown on cover slides and fixed with 4% paraformaldehyde, followed by permeabilization
128 with 0.1% Triton X-100 at room temperature. Staining was performed using anti-IKK β (Millipore,
129 diluted 1:400) primary antibody and Alexa Fluor 488-coupled secondary antibody. DNA was stained
130 with 4',6-diamidino-2-phenylindole (DAPI) by using the VectaShield mounting medium (H-1200,
131 Vector). Immunofluorescence staining was visualized with a Nikon Eclipse Ti inverted microscope
132 (with cooled LED excitation system).

133

134 **Immunoblotting and antibodies**

135 For the preparation of protein extracts, cellular pellets were resuspended in lysis buffer (20 mM Tris-
136 HCl pH 8.0, 200 mM NaCl, 0.5% Nonident P40, 1 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 1 mM
137 PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin). After quantification (Interchim BC Assay), equal amounts

138 of proteins were separated by electrophoresis on SDS-polyacrylamide gel, and then transferred onto a
139 Polyscreen PVDF membrane in a Trans-Blot electrophoretic transfer cell (Bio-Rad) and subjected to
140 immunoblotting (IB). Immunoblotting for histones was performed by the lysis of cellular pellets for 10
141 minutes in ice with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium.deoxycholate,
142 1% Triton X-100) supplemented with protease inhibitor tablet (Roche). The cellular extracts were
143 sonicated twice for 10 seconds (30% amplitude) and centrifuged for 10 minutes at 4°C at 11,000 g. Then
144 40 µg of protein lysate was subjected to western blotting as described above.

145 Nuclear extracts were prepared using the Panomics nuclear extraction kit (catalog no. AY2002)
146 following the manufacturer's instructions. The following antibodies were used for IB: anti-IKKβ (2684;
147 Cell Signaling), anti-p73 (Ab-1; Calbiochem), anti-EZH2 (AC22; Cell Signaling), anti-histone H3
148 (9715; Cell Signaling), anti-H3Lys27m3 (4039; Epigentek), anti-DNMT1 (clone 60B1220; Abnova),
149 anti-β-actin (C4; MP Biomedicals), anti-β-tubulin, anti-PARP (9542; Cell Signaling), anti-Flag (F4042,
150 Sigma-Aldrich) and anti-HA (3F10; Roche). Images were taken using the ChemiDoc XRS+ imaging
151 system (Bio-Rad).

152

153 **Chromatin immunoprecipitation**

154 Chromatin immunoprecipitation (ChIP) assay was performed using the Shearing ChIP and OneDay
155 ChIP kits (Diagenode, Philadelphia, PA, USA) according to the manufacturer's instructions. Briefly,
156 cells were sonicated to obtain DNA fragments of 200-500 bp. Sheared chromatin was
157 immunoprecipitated with isotype control IgG or the following antibodies: anti-p73 (Ab-1; Calbiochem),
158 anti-IKKβ, anti-EZH2 (AC22; Cell Signaling), anti-DNMT1 (clone 60B1220; Abnova) and anti-
159 H3Lys27m3 (4039; Epigentek). After DNA recovery, 1% of input chromatin and the precipitated DNA

160 were analyzed by qPCR with the primers listed in Table 1. The CHIP results are presented in the
161 histograms as the percentage of binding relative to the input (100%).

162 For CHIP/Re-CHIP experiments, bead-bound protein-DNA complexes obtained after the first CHIP were
163 incubated with 50 μ l of 10 mM DTT for 30 minutes at 37°C, shaking at 300 rpm. Supernatant was
164 collected after centrifugation at 12,000 g for 1 minute. Pelleted beads were incubated again with 30 μ l of
165 10 mM DTT for 20 minutes and centrifuged at 12,000 g for 1 minute. Ten percent of the combined
166 supernatants were kept as input of the second CHIP, which was performed following the OneDay CHIP
167 kit (Diagenode) manufacturer's protocol.

168

169 **Oligonucleotide pull-down**

170 Cells were lysed and sonicated in HKMG buffer (10 mM HEPES pH 7.9, 100 mM KCl, 5 mM MgCl₂,
171 10% glycerol, 1 mM DTT and 0.5% NP-40) containing protease and phosphatase inhibitors. After
172 centrifugation at 12,000 g for 10 minutes, protein extracts was pre-cleared with streptavidin-agarose
173 beads. The PIG3 promoter was used as a template to amplify the p53RE region containing wild-type or
174 mutated p53RE. PCR amplification was performed using a biotinylated forward primer (5'-Btm-
175 CCCAGGACTGCGTTTTGCCT-3') and non-biotinylated reverse primer (5'-
176 GGTCCATTTCCAGGCATGG-3'). Amplicons were extracted from agarose gel using the MinElute
177 Gel Extraction kit (Qiagen) and quantified. Then, 2 mg of prepared protein extract was incubated with 1
178 μ g of biotin-PIG3 promoter probes and 10 μ g of poly(dI-dC).poly(dI-dC) for 16 h at 4°C. DNA-bound
179 proteins were collected with streptavidin-agarose beads for 1 h and washed 5 times with HKMG buffer.
180 DNA-bound proteins were then analyzed by IB.

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182 **Dual-luciferase reporter assay**

183 38E6E7 HFK were transfected with firefly luciferase pGL3-PIG3 promoter (p53REwt or p53REm)
184 vector (0.5 µg) and pRL-TK Renilla reporter vector (15 ng) used as an internal control. Cells were lysed
185 after 48 h, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System
186 (Promega). Light emission was measured with an Optocompt luminometer (MGM Instruments).
187 Relative expression of firefly luciferase to Renilla luciferase was compared between p53REwt and
188 p53REm PIG3 promoters and expressed in relative luminescence units (RLU).

189

190 **Pyrosequencing**

191 Genomic DNA from cell lines and primary cells was prepared by proteinase K treatment, salting out
192 extraction and isopropanol precipitation. Sodium bisulfite modification was performed on 500 ng of
193 DNA using the EZ DNA Methylation-Gold Kit (Zymo Research). Quality and completeness of DNA
194 modification was assessed using control primers for modified and non-modified DNA, respectively
195 (Table 2). To quantify the percentage of methylated cytosine in individual CpG sites, bisulfite-converted
196 DNA was sequenced using a pyrosequencing system (PSQ 96MA, Biotage, Sweden). This method treats
197 each individual CpG site as a C/T polymorphism and generates quantitative data for the relative
198 proportion of methylated versus unmethylated allele. Hot-start PCR was performed with the HotStarTaq
199 Master Mix kit (Qiagen), and pyrosequencing was carried out in accordance with the manufacturer's
200 protocol (Biotage). The target CpGs were evaluated by converting the resulting pyrograms into
201 numerical values for peak heights.

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203 **Statistical analysis**

204 Variations of sample values are represented by error bars that show the standard deviation from the
205 mean. The statistical differences were analyzed using a two-sided Student t-test. The p values were
206 considered significant if $p < 0.05$ (*); p values below 0.01 were considered highly significant (**).

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226 **Results**

227 **IKK β nuclear localization and Δ Np73 α accumulation are mediated mainly by HPV38 E7**
228 **oncoprotein**

229 Our previous work showed that I κ B kinase beta (IKK β), known to play a key role in the cytoplasm as a
230 positive regulator of the NF- κ B signaling pathway, is also localized in the nucleus of human foreskin
231 keratinocytes (HFK) immortalized by the E6 and E7 oncoproteins of beta HPV38 (38E6E7 HFK) (14).
232 In these cells, the IKK β nuclear form phosphorylated and stabilized Δ Np73 α , which in turn inhibited
233 the expression of some p53-regulated genes (14). To corroborate our findings and demonstrate that
234 IKK β nuclear accumulation was dependent on the expression of HPV38 E6 and E7, we generated
235 HPV38 immortalized HFK lines in which we could silence the expression of viral genes. HFK were
236 transduced by recombinant retroviruses in which HPV38 E6 and E7 genes are flanked by two *LoxP*
237 sites. After immortalization, 38E6E7 HFK were transduced with a retrovirus expressing the recombinase
238 Cre, which resulted in the deletion of the viral genes and consequent loss of its expression. Real-time
239 PCR showed a decrease of approximately 50% in HPV38 E6 and E7 mRNA levels (Figure 1A), which
240 resulted in a significant reduction in IKK β in the nucleus as determined by immunofluorescence and
241 cellular fractionation (Figures 1B and C).

242 Next, we determined whether IKK β nuclear translocation and Δ Np73 α accumulation were induced by
243 one or both viral oncoproteins and generated HFK expressing HPV38 E6 (38E6 HFK) or HPV38 E7
244 (38E7 HFK) (Figure 1D). Immunofluorescence staining showed that IKK β is localized mainly in the
245 cytoplasm in HFK and 38E6 HFK, whereas it is accumulated in the nucleus of 38E7 HFK (Figure 1E).
246 Accordingly, Δ Np73 α protein levels were elevated in 38E7 HFK compared with 38E6 HFK and mock
247 cells (Figure 1F). In addition, as expected, inhibition of IKK β by the chemical compound Bay-11-7082
248 led to a significant decrease in Δ Np73 α protein levels (data not shown).

249 Thus, HPV38 E7 is directly involved in IKK β nuclear translocation and the resulting Δ Np73 α
250 accumulation.

251

252 **IKK β and Δ Np73 α are part of the same transcriptional regulatory complex**

253 Previous findings highlighted a nuclear role for IKK β being directly involved in transcriptional
254 regulation (28). Indeed, in the nucleus of B cells, IKK β is recruited together with the receptor of B-cell
255 activating factor (BAFF) to specific promoters, where it phosphorylates histone H3, inducing chromatin
256 remodeling and transcription initiation (28). Our previous study showed that IKK β induces the
257 recruitment of Δ Np73 α to p53-regulated promoters, but did not rule out the possibility that IKK β could
258 be part of a transcriptional regulatory complex and bind the DNA. Thus, we first determined whether,
259 similarly to Δ Np73 α , IKK β is recruited to p53-regulated promoters. Chromatin immunoprecipitation
260 (ChIP) experiments showed that in 38E6E7 HFK, IKK β is able to bind the -240/-400 region of the PIG3
261 promoter, which comprises a p53RE (Figure 2A), whereas no binding was observed on the same
262 promoter in primary keratinocytes. In addition, silencing of viral gene expression by shRNA (Figure 2B)
263 significantly affected the recruitment of IKK β and Δ Np73 α to the PIG3 promoter (Figure 2A) and
264 restored PIG3 expression (Figure 2C) as a consequence of the loss of the negative regulatory complex
265 on the promoter. ChIP/Re-ChIP experiments confirmed that IKK β and Δ Np73 α are part of the same
266 transcriptional regulatory complex (Figure 2D). To corroborate these findings, we performed oligo pull-
267 down experiments using biotinylated DNA probes that contain a region of the PIG3 promoter
268 encompassing the wild-type or mutated p53RE (the -240/-400 region). 38E6E7 HFK were transiently
269 transfected with vectors expressing Flag-IKK β or hemagglutinin (HA)-tagged Δ Np73 α , and cellular
270 extracts were prepared and used for the oligo pull-down experiments. We observed that the PIG3
271 promoter fragment containing the wild-type p53RE co-precipitated with Flag-IKK β and HA-

272 Δ Np73 α , whereas mutation of the p53RE significantly decreased precipitation of the two cellular
273 proteins (Figure 3A). Similar results were obtained using cellular extracts of 38E6E7 HFK expressing
274 endogenous levels of IKK β and Δ Np73 α (Figure 3C).

275 Due to the inhibitory function of IKK β and Δ Np73 α on the PIG3 promoter, we next determined whether
276 enzymes inducing epigenetic changes were also present in the complex. Therefore, we analyzed the
277 precipitated complexes in oligo pull-down assays by IB using several antibodies against epigenetic
278 enzymes (data not shown). The IKK β / Δ Np73 α complex co-precipitated with the Polycomb-group (PcG)
279 2 member EZH2 and DNA methyltransferase DNMT1 (Figure 3B). As observed for IKK β and Δ Np73 α ,
280 mutation of the PIG3 53RE affected precipitation of EZH2 and DNMT1 (Figure 3B). As control, we
281 also determined the presence of p65 since this promoter region used in the assay also includes a putative
282 NF- κ B RE. As expected, p65 binding to the DNA probe was not affected by mutation of the p53RE
283 (Figure 3B). Finally, transient transfection experiments in 38E6E7 HFK using constructs containing the
284 PIG3 promoter cloned in front of the Luciferase reporter gene showed that mutation of the p53RE
285 strongly increased Luciferase activity (Figure 3D), providing additional evidence for the transcriptional
286 inhibitory role of the p53RE in these cells.

287 To further evaluate the possibility that the four cellular proteins are part of the same complex, we
288 performed CHIP/Re-CHIP experiments. We first carried out CHIP with DNMT1, EZH2 or Δ Np73 α
289 antibodies, followed by Re-CHIP using an IKK β antibody. The data showed that DNMT1, EZH2 and
290 Δ Np73 α can associate with IKK β (Figure 4A), further confirming the data obtained with the oligo pull-
291 down assay shown in Figure 3A and B. CHIP experiments carried out in cells expressing either HPV38
292 E6 or E7 showed that DNMT1, EZH2, Δ Np73 α and IKK β bind to the PIG3 promoter in the presence of
293 E7, but not E6 (Figure 4B), in agreement with previous results showing that HPV38 E7 induces high
294 levels of Δ Np73 α and IKK β nuclear accumulation (Figure 1E and F). In addition, down-regulation of

295 Δ Np73 α expression in 38E6E7 HFK by transfection of Δ Np73 α AntiSense oligonucleotide (AS) led to
296 loss of the tetrameric complex on the PIG3 promoter (Figure 4C). These results were consistent with
297 previous findings from our group showing that Δ Np73 α AS treatment of 38E6E7 HFK led to rescue of
298 the mRNA levels of different p53-regulated genes, including *PIG3* (3). We also observed that 38E6E7
299 HFK displayed increased levels of EZH2 compared with primary keratinocytes (Figure 4D). However,
300 total trimethylation at lysine 27 of histone H3 (H3Lys27m3), an event induced by EZH2, did not
301 significantly change in the presence of HPV38 E6 and E7 (Figure 4E), showing that the events described
302 here are not the results of a general effect of HPV38 E6 and E7 on the enzymatic activity of EZH2, but
303 are specific for a subset of gene promoters.

304 Together, these findings demonstrate the existence in 38E6E7 HFK of a tetrameric complex able to
305 inhibit the expression of p53-regulated genes, such as *PIG3*.

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307 **The IKK β / Δ Np73 α /DNMT1/EZH2 tetrameric complex induces epigenetic changes on the PIG3** 308 **promoter, inhibiting its activity**

309 We further characterized the role of this novel tetrameric complex in transcriptional regulation. ChIP
310 experiments revealed H3Lys27 hypermethylation in the PIG3 promoter region surrounding the p53RE
311 as well as in the PIG3 coding sequence region (+322/+492), which contains a microsatellite region that
312 has been proposed as a p53 binding site (29) (Figure 5A). Interestingly, H3Lys27m3 was not observed
313 in the promoter of another p53-regulated gene, Survivin (Figure 5A), indicating that the IKK β / Δ Np73 α
314 complex does not bind to the p53RE of all p53-regulated promoters. To further explore this issue, we
315 performed ChIP for IKK β , Δ Np73 α , DNMT1 and EZH2 and investigated their ability to bind to
316 different p53-regulated promoters. The complex was found to be associated with most of the examined
317 p53-regulated promoters, except the Survivin promoter (Figure 5B).

318 Next, we evaluated the role of the DNA methyltransferase DNMT1 in silencing PIG3 expression. The
319 PIG3 promoter is located in a CpG-rich region containing the p53RE 320 bp upstream of the
320 transcription start site (TSS). This CpG-rich region also includes a bona fide CpG island overlapping the
321 first exon of the gene containing the microsatellite region (Figure 5C). Pyrosequencing assays were
322 performed in bisulfite-modified DNA obtained from HFK as well as 38E6E7 HFK cultured in the
323 presence or absence of 5-Aza. Our analysis revealed that the CpGs on the PIG3 promoter region around
324 the p53RE are highly methylated in 38E6E7 HFK (close to 100%) and less methylated in mock cells
325 (HFK) (average, 46%). No methylation was observed at the level of the microsatellite region and the
326 TSS (Figure 5C). Intermediate methylation levels were obtained after 5-Aza treatment of 38E6E7 HFK.
327 In accordance with this observation, exposure of 38E6E7 HFK to DNA 5-Aza led to the rescue of PIG3
328 expression (Figure 5D). The inhibition of DNMT1 intracellular levels in 38E6E7 HFK by specific
329 siRNA also resulted in increased PIG3 expression (Figure 5E), confirming the specificity of the results
330 obtained with 5-Aza treatment. Gene silencing of EZH2 by siRNA also led to a rescue of PIG3
331 expression levels (Figure 5E). In addition, knock-down of either DNMT1 or EZH2 resulted in inhibition
332 of 38E6E7 HFK growth ability (Figure 5F). In summary, the epigenetic enzymes EZH2 and DNMT1,
333 via their interaction with the $\Delta Np73\alpha$ /IKK β complex, appear to be directly involved in the repression of
334 p53-regulated promoters inducing H3Lys27m3 and DNA methylation, respectively, and play a positive
335 role in cellular proliferation.

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337 **IKK β plays a key role in the proliferation of 38E6E7 HFK independent of NF- κ B signaling**

338 Our data highlighted a novel role of IKK β in the nucleus of 38E6E7 HFK. In addition, we previously
339 showed that HPV38 E6 and E7 stimulate IKK β activity in the cytoplasm, promoting I κ B α
340 phosphorylation and degradation, leading to activation of NF- κ B signaling (14, 30). We therefore

341 determined whether IKK β could contribute to proliferation of 38E6E7 HFK, independent of the
342 activation of NF- κ B signaling. We first stably expressed in 38E6E7 HFK a deletion mutant of I κ B α
343 (Δ N-I κ B α) that lacks the first 36 amino acids at the N terminus encompassing the IKK β
344 phosphorylation sites. Δ N-I κ B α is no longer regulated by IKK β and is able to constitutively inhibit the
345 NF- κ B canonical pathway by sequestering p65 in the cytoplasm (14, 30). As shown by colony formation
346 assays and short-term growth curves, inhibition of the NF- κ B canonical pathway only marginally
347 affected the proliferation of 38E6E7 HFK (Figure 6A and 6B). In contrast, constitutive expression of a
348 dominant-negative mutant of IKK β (Flag-DN-IKK β) in 38E6E7 HFK significantly decreased their
349 proliferation (Figure 6C and D). Similar experiments performed in HFK showed that blocking IKK β ,
350 but also NF- κ B negatively affected their growth ability (data not shown). Taken together, these data
351 show that IKK β , independently of the NF- κ B signaling, plays an additional role in promoting
352 proliferation. However, in 38E6E7 HFK the stronger effect of DN-IKK β on proliferation in comparison
353 to Δ N-I κ B α is partly explained by the ability of IKK β to promote Δ Np73 α accumulation in the nucleus
354 (14), a an event that does not occur in primary HFKs.

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364 **Discussion**

365 Several viruses are able to inactivate p53 functions, probably as part of their strategies to guarantee the
366 completion of the viral life cycle. Mucosal high-risk HPV types induce p53 degradation via the E6
367 oncoprotein. Although beta HPV49 has the same mechanism as the mucosal high-risk HPV types in
368 inactivating p53 (31), other beta HPV types target this cellular tumor suppressor by alternative strategies
369 (3, 7, 9, 14). We previously showed that expression of beta HPV38 E6 and E7 oncoproteins in primary
370 keratinocytes promoted accumulation of the p53 antagonist $\Delta\text{Np73}\alpha$ and cellular immortalization (3, 4,
371 6, 14). Down-regulation of $\Delta\text{Np73}\alpha$ expression in 38E6E7 HFK significantly reduced cellular viability
372 and resulted in activation of p53-dependent apoptosis (3). Similarly, knocking down $\Delta\text{Np73}\alpha$ expression
373 in HPV38 E6 and E7 transgenic mice partially restored p53 functions (15). Thus, data obtained in *in*
374 *vitro* and *in vivo* experimental models highlighted the key role of $\Delta\text{Np73}\alpha$ accumulation in cellular
375 proliferation and transformation.

376 One of the key events in $\Delta\text{Np73}\alpha$ accumulation in 38E6E7 HFK is the translocation of IKK β into the
377 nucleus, where it phosphorylates and stabilizes $\Delta\text{Np73}\alpha$ (14). Here, we show that these events are
378 mediated by HPV38 E7. The underlying mechanism is still unknown. Several viruses have been
379 demonstrated to interfere with the NF- κ B pathway (32, 33); for instance, HTLV1 is known to activate
380 the IKK complex by direct binding of its transforming protein, Tax, to IKK γ (34). Preliminary studies
381 from our group have shown that, similarly to Tax, HPV38 E7 can bind to IKK β (Sadij, unpublished
382 data). Whether this event triggers nuclear accumulation of IKK β is still under investigation.
383 Interestingly, independent studies have described novel IKK β functions in the nucleus, although none of
384 them were linked to $\Delta\text{Np73}\alpha$ (35-37). For instance, Fu *et al.* reported that IKK β forms a positive
385 transcriptional regulatory complex with the receptor of B-cell activating factor (BAFF) (28). Findings of
386 the current study show that, similarly to the BAFF complex, IKK β is intrinsically part of the

387 transcriptional regulatory complex that inhibits the expression of some p53-regulated genes. Most
388 importantly, our data showed for the first time that two epigenetic enzymes, EZH2 and DNMT1, are part
389 of the IKK β / Δ Np73 α complex. EZH2 is a component of the Polycomb complex 2 and induces
390 H3Lys27m3 and consequent closure of chromatin (38). Accordingly, ChIP experiments performed in
391 38E6E7 HFK demonstrated the presence of H3Lys27m3 of p53-regulated promoters.

392 The presence of DNMT1 in the IKK β / Δ Np73 α complex is consistent with previous findings that
393 documented its ability to bind to EZH2 (38) and can account for the high levels of methylation detected
394 in the region surrounding the p53RE in 38E6E7 HFK. EZH2 and DNMT1 binding to the IKK β / Δ Np73 α
395 complex could contribute to its ability to block p53-mediated transcription. According to the current
396 model, Δ Np73 α exerts its dominative negative function toward p53 via competition for the same RE, as
397 the two proteins share a homologous DNA binding domain (39). Due to its lack of a transactivation TA
398 domain, binding of Δ Np73 α to the promoter of certain genes results in their transcriptional silencing
399 (16, 17). Our previous data confirm this model; in fact, we showed that in 38E6E7 HFK, Δ Np73 α bound
400 to the p53RE in the PIG3 promoter, and that silencing of Δ Np73 α expression restored p53 binding to the
401 promoter and re-activated PIG3 expression (3, 14).

402 Consistent with the previous findings, in the present study we observed that the “naked” DNA construct,
403 containing the Luciferase gene driven by the wild-type PIG3 promoter, has low activity if transfected
404 into 38E6E7 HFK, whereas it can be strongly activated if Δ Np73 α binding to p53RE is hampered.
405 However, here we described that Δ Np73 α is able to repress transcription also by facilitating the
406 recruitment of DNMT1 and EZH2 to specific p53-regulated promoters, which in turn induce epigenetic
407 changes. It is likely that these two enzymes, together with Δ Np73 α and IKK β , contribute to prolonged
408 silencing of the expression of some p53-regulated genes under certain conditions, for instance during
409 cellular transformation. Indeed, EZH2 and DNMT1, like Δ Np73 α , are often up-regulated in cancer (40-

410 44), where they appear to target specific genes for methylation (45). In addition, our data show that
411 silencing the expression of EZH2 and DNMT1 in 38E6E7 HFK leads to a rescue of PIG3 levels and to a
412 reduction in cell growth ability, further confirming the key role of EZH2 and DNMT1 in HPV38-
413 mediated inhibition of p53-regulated expression.

414 The mechanisms by which EZH2 and DNMT1 can be recruited to specific promoters are still poorly
415 characterized (46). Our findings show that $\Delta Np73\alpha$ is one of these mechanisms. Indeed, down-
416 regulation of $\Delta Np73\alpha$ in 38E6E7 HFK led to disruption of the complex and release of the two epigenetic
417 enzymes, which are no longer recruited to the PIG3 promoter.

418 We previously showed that $\Delta Np73\alpha$ and IKK β co-localize in the nucleus of cancer-derived cells, where
419 they are responsible for inhibition of p53 pro-apoptotic pathways (14). Down-regulation of $\Delta Np73\alpha$ or
420 IKK β levels in these cancer cells led to an increase in their sensitivity to chemo-therapeutic agents by
421 restoring p53-mediated apoptosis (our unpublished data). As EZH2 and DNMT1 are both found to
422 accumulate in cancer cells, it will be of interest to explore whether, as observed in 38E6E7 HFK, they
423 can form a multimeric complex with $\Delta Np73\alpha$ and IKK β in cancer-derived cell lines in order to
424 constitutively silence p53-regulated transcription.

425 In summary, here we show for the first time that the E7 oncoprotein of HPV38 promotes the formation
426 of a multimeric complex that negatively regulates the expression of some p53 target gene promoters.
427 Whether this is a specific event exclusively occurring in HPV38-infected cells or a more common
428 mechanism taking place in human carcinogenesis remains to be further investigated.

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635 **Figure legends**

636 **Figure 1. HPV38 E7 expression promotes IKK β nuclear accumulation**

637 (A, B and C) HFK were first transduced with the retrovirus pBabe-puro-38E6E7^{LoxP/LoxP}. After selection
 638 in puromycin, cells were transduced with retrovirus carrying either pBabe-hygro-CRE recombinase or
 639 pBabe-hygro as negative control and selected for hygromycin resistance. The results were compared
 640 with the parental untransduced HFK.

641 (A) Total RNA from indicated control HFK and transduced HFK was extracted, and HPV38 E6 and E7
 642 expression levels were measured by reverse transcription quantitative PCR (RT-qPCR). Data shown are
 643 the mean values of three independent experiments. **p<0.01 pB-hygro-CRE vs. the respective pBabe-
 644 hygro control sample.

645 (B) HFK, pBabe-hygro-CRE and pBabe-hygro HFK were seeded on cover slides and stained with anti-
 646 IKK β antibody. Cellular nuclei were counterstained with DAPI. Results are representative of two
 647 independent experiments.

648 (C) Twenty micrograms of nuclei extracts and 20 μ g of total lysate from HFK, pBabe-hygro-CRE and
 649 pBabe-hygro HFK were analyzed by immunoblotting with the indicated antibodies (left panel). Protein
 650 bands were quantified using Bio-Rad Image Lab software (right panel). The histogram represents the
 651 levels of IKK β in the total and nuclear extract after normalization to the levels of β -tubulin and PARP,
 652 respectively. Data shown are the mean values of three independent experiments. **p<0.01 pB-hygro-
 653 CRE vs. the pB-hygro control sample.

654 (D, E and F) HFK expressing HPV38 E6 (38E6 HFK) or HPV38 E7 (38E7 HFK) were generated by
 655 retroviral transduction. HFK transduced with the pLXSN empty vector (HFK) were used as negative
 656 control. After transduction, HFK were selected in neomycin, harvested and processed for the preparation
 657 of total RNA and protein extracts.

658 (D) The expression of HPV38 E6, E7 and GAPDH was determined by RT-PCR. Results are
 659 representative of two independent experiments.

660 (E) HFK, 38E6 HFK and 38E7 HFK were seeded on cover slides and stained with anti-IKK β antibody
 661 and DAPI (upper panel). In each staining, the percentage of cells with nuclear or cytoplasmic staining
 662 was determined by counting 100 cells/field in three different fields. Cells with nuclear or cytoplasmic
 663 staining for IKK β were represented as the ratio to total cell number (100%). Results shown in the
 664 histogram (lower panel) are the average of two independent experiments. The difference in the
 665 percentage of cells with nuclear IKK β in 38E7 HFK vs. HFK is significant (**p<0.01).

666 (F) Forty micrograms of total protein extract from HFK, 38E6 HFK and 38E7 HFK were analyzed by IB
 667 with the indicated antibodies. Results are representative of two independent experiments.

668

669 **Figure 2. Δ Np73 α and IKK β co-bind on the PIG3 promoter**

670 (A) HFK and 38E6E7 HFK transduced with pRetroSuper (pRS) or shRNA-HPV38E6E7 (pRS
 671 sh38E6E7) were fixed with formaldehyde and processed for ChIP using anti-IKK β , anti-p73 α and non-
 672 specific IgG. The eluted DNA was analyzed by qPCR with primers flanking the p53RE within the PIG3
 673 promoter. Part of the total chromatin fraction (1/10th) was used as input. The histogram shows the
 674 amount of promoter specifically bound by each protein and expressed as a percentage of the amount of
 675 the PIG3 promoter in the input (100%). Results shown in the histogram are the average of three
 676 independent experiments performed in duplicate. The differences in the percentage of binding between
 677 IKK β or Δ Np73 α ChIP and the corresponding IgG control are significant (**p<0.01) in pRS and pRS
 678 sh38E6E7 samples. In addition, the decrease in IKK β or Δ Np73 α binding affinity to the PIG3 promoter
 679 in pRS sh38E6E7 vs. pRS is also significant (**p<0.01).

680 (B) 38E6E7 HFK were retrovirally transduced with pRS empty vector or pRS sh38E6E7. After
 681 puromycin selection, cells were collected for RNA extraction. The mRNA levels of HPV38 E6 and E7
 682 were determined by RT-qPCR. Results are the average of three independent experiments performed in
 683 duplicate. **p<0.01 for pRS sh38E6E7 vs. the corresponding pRS sample.

684 (C) Total RNA was extracted from HFK, 38E6E7 HFK and 38E6E7 HFK transduced with pRS and pRS
 685 sh38E6E7 and analyzed by RT-qPCR for PIG3 expression levels and normalized to GAPDH levels.
 686 Results are the average of three independent experiments performed in duplicate. **p<0.01 between the
 687 indicated samples.

688 (D) 38E6E7 HFK were processed for ChIP with Δ Np73 α or IKK β antibodies. The eluted DNA was
 689 subjected to Re-ChIP with IKK β or p73 α antibodies, respectively. IgG antibody was included as
 690 negative control. Input and co-IP DNA were analyzed by qPCR for the p53RE region of the PIG3
 691 promoter. Results are the average of three independent experiments performed in duplicate. **p<0.01
 692 vs. the corresponding IgG control.

693

694 **Figure 3. EZH2 and DNMT1, like Δ Np73 α and IKK β , bind to p53RE on the PIG3 promoter**

695 (A and B) 38E6E7 HFK transiently expressing HA- Δ Np73 α and Flag-IKK β were processed for the
 696 oligo pull-down assay. Two milligrams of cell lysate was incubated with biotinylated probes containing
 697 the p53RE of the PIG3 promoter, either wild-type (p53REwt) or mutated (p53REm). DNA-associated
 698 proteins were recovered by precipitation with streptavidin beads and analyzed by IB with the indicated
 699 antibodies left panel). Protein band intensities of three independent IB for each experiment were
 700 quantified by Image Lab (Bio-Rad) and shown in the right panels. Results are the average of three

701 independent experiments. The differences in the binding affinity of IKK β and Δ Np73 α to the p53REwt
 702 vs. p53REm probes are significant (**p<0.01).

703 (C) The oligo pull-down assay was performed using 2 mg of cell lysate from untransfected 38E6E7
 704 HFK, as explained for Figure 3A and B. Results are the average of two independent experiments.
 705 *p<0.05 for p53REm vs. the corresponding p53REwt sample.

706 (D) 38E6E7 HFK were transfected with PIG3 promoter-firefly luciferase reporter construct harboring
 707 either a wild-type or mutated p53RE and with a Renilla construct. At 24 h after transfection, cells were
 708 processed for the luciferase assay. Relative light units (RLU) values are represented in the histogram.
 709 The data shown are the mean values of three independent experiments performed in triplicate. The
 710 differences in the luciferase activity between mutated and wild-type promoter are significant (**p<0.01).

711

712 **Figure 4. Endogenous EZH2, DNMT1, IKK β and Δ Np73 α co-bind on the PIG3 promoter**

713 (A) 38E6E7 HFK were harvested and formaldehyde-fixed. After sonication, the chromatin was
 714 subjected to ChIP assay using DNMT1, EZH2 or Δ Np73 α antibodies. The eluted DNA was processed
 715 for Re-ChIP with anti-IKK β antibody or non-specific IgG control. Input and co-IP DNA were analyzed
 716 by qPCR for the p53RE region of the PIG3 promoter. Results are the average of two independent
 717 experiments performed in duplicate. *p<0.05 and **p<0.01 vs. the corresponding IgG control.

718 (B) HFK, 38E6 HFK and 38E7 HFK were subjected to ChIP assay using antibodies for the indicated
 719 proteins or non-specific IgG control. Input and eluted DNA were analyzed by qPCR for the p53RE
 720 region of the PIG3 promoter. Results were generated as indicated for Figure 2A and are the average of
 721 two independent experiments performed in duplicate. *p<0.05 and **p<0.01 vs. the corresponding IgG
 722 control.

723 (C) Sense and Δ Np73 α -AntiSense oligonucleotides were transfected into 38E6E7 HFK. The cells were
 724 collected 36 h after transfection and used for ChIP assay with the indicated antibodies. The amounts of
 725 p53RE bound to the different proteins were calculated as described for Figure 2A. Data shown are the
 726 mean values of two independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. the corresponding IgG control.
 727 The protein levels of Δ Np73a and β -actin were detected by IB (top right panel). Result is representative
 728 of two independent experiments.

729 (D) Protein extracts (40 μ g) from HFK and 38E6E7 HFK were analyzed by IB for protein levels of
 730 DNMT1, EZH2 and β -actin (left panel). Band intensities were quantified and normalized to β -actin
 731 levels (right panel). Data shown are the mean values of three independent experiments. * $p < 0.05$ vs. the
 732 corresponding HFK sample.

733 (E) HFK and 38E6E7 HFK were lysed with RIPA buffer and sonicated as explained in Materials and
 734 Methods. The protein extracts (40 μ g) were analyzed by IB for protein levels of histone H3Lys27m3,
 735 total histone H3 and β -actin (left panel). Band intensities were quantified as explained for Figure 1C and
 736 normalized to β -actin levels (right panel). Data shown are the mean values of two independent
 737 experiments. The difference in the levels of histone H3Lys27m3 between 38E6E7 HFK and HFK is not
 738 statistically significant.

739

740 **Figure 5. The PIG3 promoter is silenced by DNA methylation and histone H3 Lys27**
 741 **trimethylation**

742 (A) 38E6E7 HFK were harvested and processed for ChIP using antibodies for the indicated proteins and
 743 non-specific IgG control. The bound DNA was analyzed by qPCR with primer sets specific for two
 744 regions of the *PIG3* gene (the p53RE region of the *PIG3* promoter and the *PIG3* ORF +322/+492

745 region) as well as primer for Survivin or GAPDH promoter. The amount of promoter specifically bound
746 by each protein was expressed as a percentage of the input sample. Data shown are the mean values of
747 three independent experiments performed in duplicate. ** $p < 0.01$ vs. the corresponding IgG control.

748 (B) 38E6E7 HFK were subjected to ChIP using antibodies for the indicated proteins and non-specific
749 IgG control. PCR was performed with primers flanking the p53REs of the indicated gene promoters.
750 Input indicates 10% of total DNA per IP. PCR products were loaded on 2% agarose gel. Data shown are
751 representative of two independent experiments.

752 (C) DNA of HFK, 38E6E7 HFK and 38E6E7 HFK treated with 5-Aza was extracted, and the
753 methylation status of indicated PIG3 promoter regions was analyzed by pyrosequencing. Assays were
754 designed next to the p53RE, TSS and microsatellite regions. Forward (F) and reverse (R) primers used
755 for pyrosequencing are indicated. Average methylation is shown for the different CpG sites analyzed.
756 CpG sites are named relative to the distance to TSS. Data shown are representative of two independent
757 experiments. The differences in the methylation levels in the -566/-458 region of the PIG3 promoter in
758 38E6E7 HFK vs. 38E6E7 HFK + 5-Aza, and in 38E6E7 HFK vs. HFK are statistically significant (p
759 values of 0.02 and 0.003, respectively). No statistically significant differences were observed in other
760 regions (+97/+103 and +339/+359).

761 (D) 38E6E7 HFK were treated for 72 h with DNA methylation inhibitor 5-Aza or DMSO alone
762 (Control). PIG3 mRNA levels were analyzed by RT-qPCR. Data shown are the mean values of three
763 independent experiments. * $p < 0.05$ vs. the untreated control sample.

764 (E) 38E6E7 HFK were transfected with a control siRNA (Scramble) or stealth siRNAs specific for
765 EZH2 and DNMT1. At 72 h after transfection, the protein levels of DNMT1, EZH2 and β -actin were
766 analyzed by IB (left panel), and PIG3 mRNA levels were determined by RT-qPCR (right panel). The

767 results are representative of two independent experiments. * $p < 0.05$ and ** $p < 0.01$ for siRNAs vs.
 768 Scramble sample.

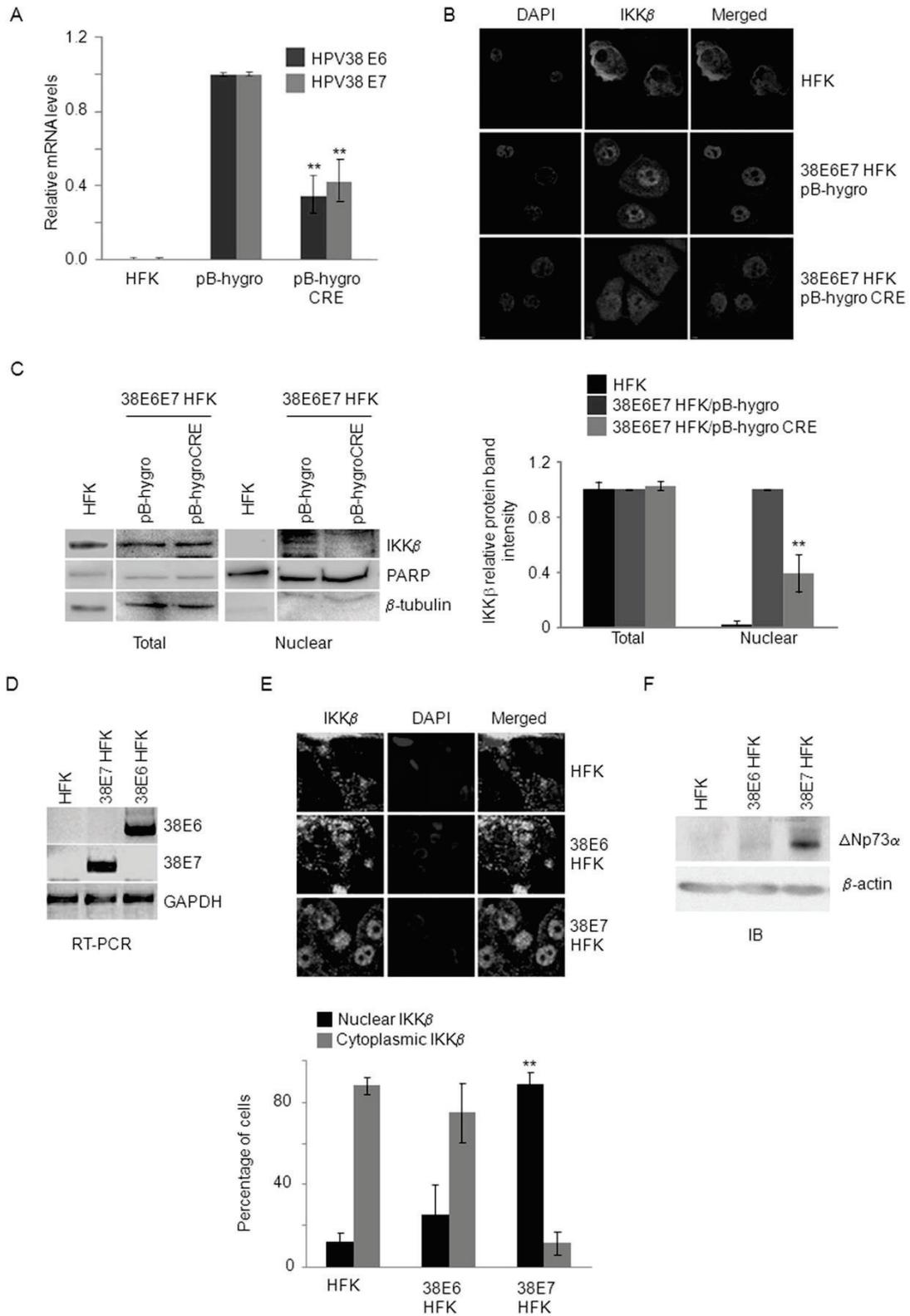
769 (F) 38E6E7 HFK were transfected with a control siRNA (Scramble) or stealth siRNAs specific for
 770 EZH2 and DNMT1. The day after transfection, 10^4 cells were seeded to perform colony formation
 771 assays. Cells were cultured for 7 days and then stained with crystal violet. Results are representative of
 772 two independent experiments.

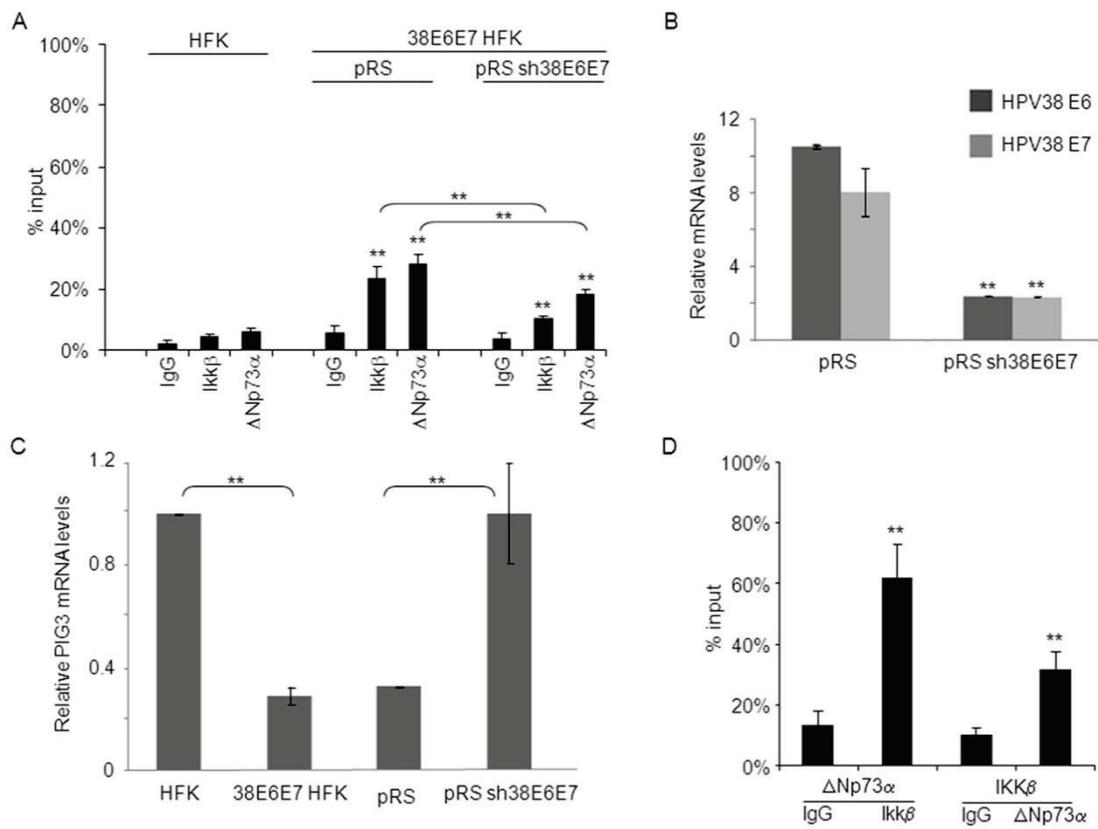
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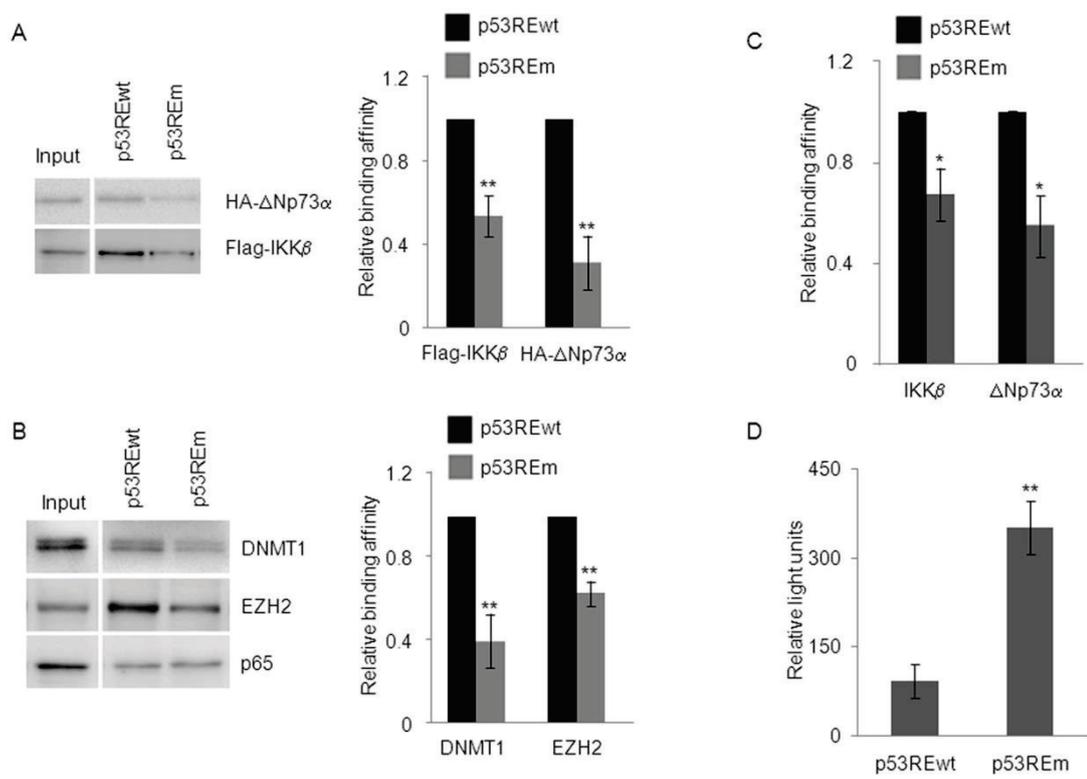
774 **Figure 6. IKK β plays an NF- κ B-independent role in 38E6E7 HFK proliferation**

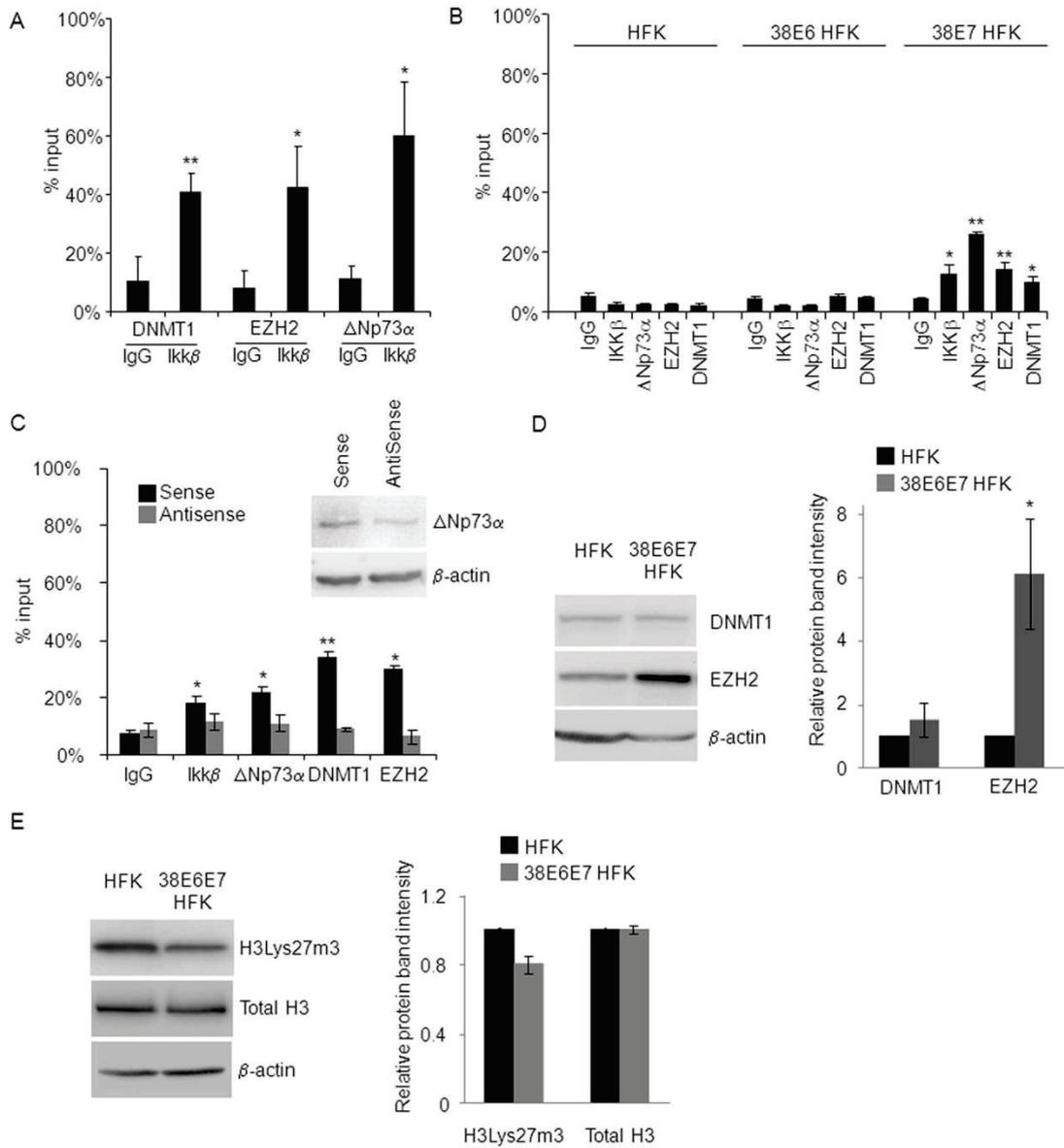
775 (A and C) 38E6E7 HFK were retrovirally transduced with pBabe-puro, pBabe-puro-dominant-negative
 776 IKK β (pB-DN-IKK β) or truncated form of I κ B α (pB- Δ N-I κ B α) and seeded at different dilution (10^4 ,
 777 10^3 and 10^2) to perform colony formation assays. Cells were cultured for 7 days and then stained with
 778 crystal violet (left panel). The average numbers of cells forming colonies were counted and reported in
 779 the histograms (right panel). Data shown are the mean values of three independent experiments.
 780 ** $p < 0.01$ 38E6E7 HFK pB-DN-IKK β vs. 38E6E7 HFK pB sample.

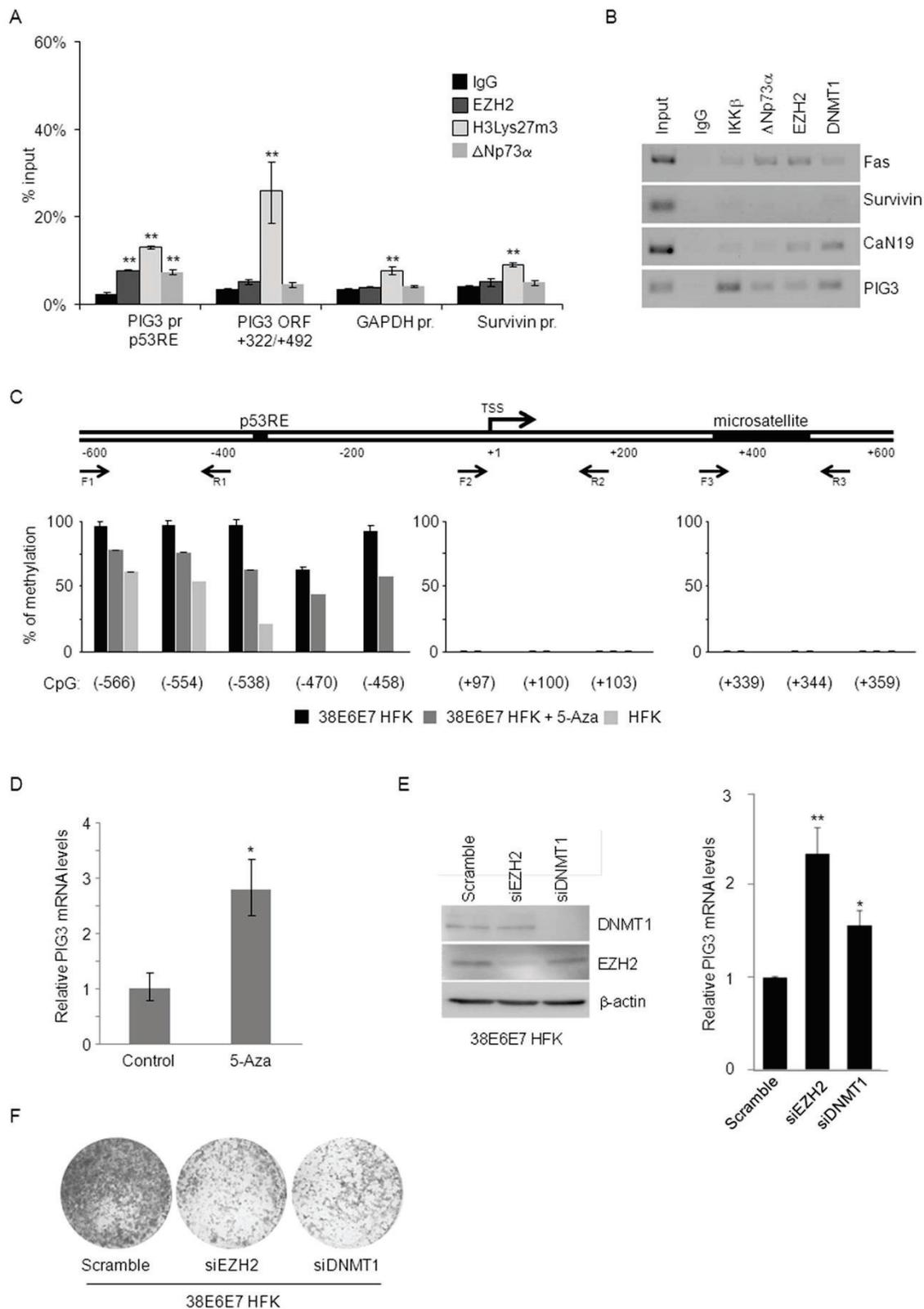
781 (B and D) After retroviral transduction, 1×10^4 38E6E7 HFK expressing DN-IKK β , Δ N-I κ B α or with
 782 pBabe control vector were seeded (day 0) and cultured for 9 days. The total numbers of cells were
 783 counted at the indicated time points and reported in the growth curve. Data shown are the mean values
 784 of three independent experiments.











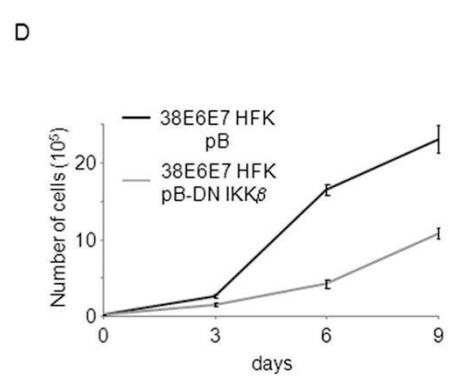
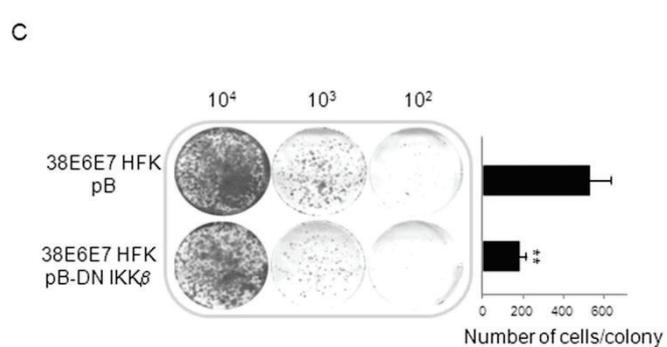
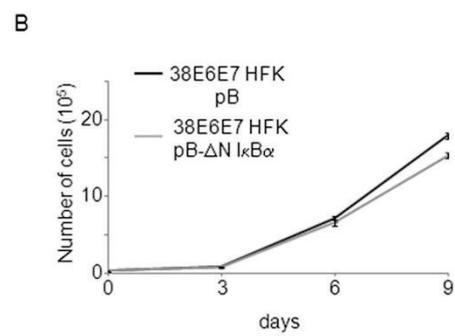
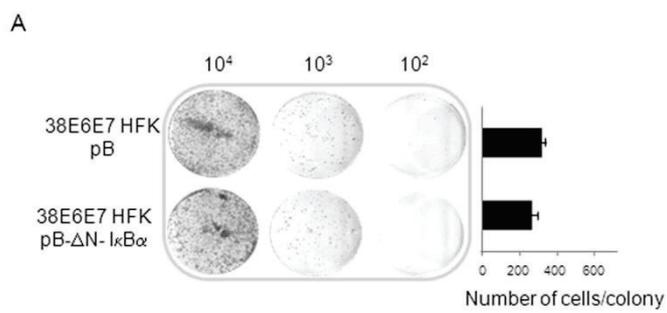


Table 1

cDNA		Primers used for RT-QPCR
PIG3	Forward	5'-GCTTCAAATGGCAGAAAAGC-3'
	Reverse	5'-AACCCATCGACCATCAAGAG-3'
HPV38 E6	Forward	5'-TCTGGACTCAAGAGGATTTTG-3'
	Reverse	5'-CACTTTAAACAATACTGACACC-3'
HPV38 E7	Forward	5'-CAAGTACTCTTCGTGATATAGTT-3'
	Reverse	5'-CAGGTGGGACACAGAAGCCTTAC-3'
GAPDH	Forward	5'-AAGGTGGTGAAGCAGGCCGT-3'
	Reverse	5'-GAGGAGTGGGTGTCGCTGTT-3'
Gene Promoters		Primers used for ChIP-QPCR
PIG3 -240/-399	Forward	5'-CCCAGGACTGCGTTTTGCCT-3'
	Reverse	5'-GGTCCATTTCCAGGCATGG-3'
PIG3 +322/+517	Forward	5'-CAGGAGCTCGGGGCGGA-3'
	Reverse	5'-ACAGGGCAGGGCAGGGC-3'
Survivin	Forward	5'-TGGGTGCCCCGACGT-3'
	Reverse	5'-GAAGGGCCAGTTCTTGAATGTAGA-3'
FasL	Forward	5'-GATCCCGCTGGGCAGGC-3'
	Reverse	5'-GTTCTGAAGGCTGCAGGCTC-3'
p21waf1	Forward	5'-TCACCATTCCCCTACCCCATGCTGCTC-3'
	Reverse	5'-AAGTTTGCAACCATGCACTTGAATGTG-3'
CaN19	Forward	5'-GGTCCAGGATGCCAGTC-3'
	Reverse	5'-GAAGGAGAGCAAGGCAGC-3'
GAPDH	Forward	5'-CACTGGAGCCTTCATCTCAG-3'
	Reverse	5'-CTGTTGCTGGCCAGCAACTG-3'

Table 2

Pyrosequencing		Primers used for PCR and sequencing (seq)
PIG3 pyro -566/-458		
Forward		5'-GGTATGGTTAGGTTTTTGGT-3'
Reverse		5'-CCAAAAATACTACTAAACATCCTAC-3'
Seq1		5'-GGAGAAGGATGTTGG-3'
Seq2		5'-AAGGTTTTTTAATTT-3'
Seq3		5'-TGGATTAGTTAGTT-3'
PIG3 pyro +97/+103		
Forward		5'-GTTGGATGYGGTAGAGTAGGAT-3'
Reverse		5'-TTCTACTTAACCCACCCC-3'
Seq1		5'-TGGAAGTTTTTGTAGT-3'
Seq2		5'-TTTTATGTATTAGG-3'
Seq3		5'-GGTTTTGGTTTTTGTG-3'
PIG3 pyro +339/+359		
Forward		5'-GAGGGAGTGTGATTTGTTTG-3'
Reverse		5'-CAACCCAACCTCAAACCTAAC-3'
Seq1		5'-TTTGATTTTTTTT-3'
Seq2		5'-TATTTTATTTTTT-3'
Seq3		5'-TAGGTAGGAGTT-3'

Chapter II: Results 2

NF- κ B Protects Human Papillomavirus Type 38 E6/E7-Immortalized Human Keratinocytes against Tumor Necrosis Factor Alpha and UV-Mediated Apoptosis

NF- κ B Protects Human Papillomavirus Type 38 E6/E7-Immortalized Human Keratinocytes against Tumor Necrosis Factor Alpha and UV-Mediated Apoptosis[∇]

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Constitutive activation of NF- κ B signaling is a key event in virus- and non-virus-induced carcinogenesis. We have previously reported that cutaneous human papillomavirus type 38 (HPV38) displays transforming properties in *in vitro* and *in vivo* experimental models. However, the involvement of NF- κ B signaling in HPV38-induced cell growth transformation remains to be determined. In this study, we showed that HPV38 E6 and E7 activate NF- κ B and that inhibition of the pathway with the I κ B α superrepressor sensitizes HPV38E6E7-immortalized human keratinocytes to tumor necrosis factor alpha (TNF- α)- and UVB radiation-mediated apoptosis. Accordingly, inhibition of NF- κ B signaling resulted in the downregulation of NF- κ B-regulated antiapoptotic genes, including cIAP1, cIAP2, and xIAP genes. These findings demonstrate a critical role of NF- κ B activity in the survival of HPV38E6E7-immortalized human keratinocytes exposed to cytokine or UV radiation. Our data provide additional evidence for cooperation between beta HPV infection and UV irradiation in skin carcinogenesis.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that infect epithelial cells of different anatomical regions (16). More than 100 HPV types have been isolated so far, and they can be classified as mucosal or cutaneous types according to their abilities to infect the mucosa of the genital and upper respiratory tracts or the skin, respectively. A subgroup of mucosal HPVs, referred to as high-risk (HR) HPV types, are the causal agents for the development of cervical cancer and other anogenital malignancies (16, 43). A vast number of studies on the HR HPV type 16 (HPV16) and others have clearly shown that the products of two early genes, E6 and E7, play a critical role in HPV-mediated carcinogenesis by altering the regulation of key cellular events, such as the cell cycle, DNA repair, apoptosis, and senescence (41). HPV16 E6 and E7 exert their transforming activities by targeting the products of tumor suppressor genes. E6 promotes the degradation of p53, affecting DNA repair and apoptosis, while E7 inactivates retinoblastoma protein (pRb) and its related proteins p107 and p130, leading to cell cycle deregulation (37, 42).

In addition to the mucosal HR HPV types, the cutaneous

HPV types belonging to the genus beta of the HPV phylogenetic tree are also suspected to play a role in the development of human cancer (16). The beta HPV types were initially isolated from nonmelanoma skin cancers (NMSCs) of patients affected by a cancer-prone genetic disease called epidermodysplasia verruciformis (EV) (47). However, it is now clear that these HPV types can also be found in NMSCs of non-EV patients, in particular in immunosuppressed individuals (6). Similar to the HR mucosal HPV types, E6 and E7 from certain beta HPV types target p53- and pRb-regulated pathways and display transforming activities in *in vitro* and *in vivo* experimental models (1, 10, 17, 21, 38, 40, 48, 51). In particular, E6 and E7 from the beta HPV38 are able to immortalize the human keratinocytes that are the natural hosts of the virus (1, 10, 21).

NF- κ B is composed of a homo- or heterodimer of proteins that belong to the NF- κ B/REL family of transcription factors (27), and NF- κ B can be activated in two distinct pathways, referred to as the canonical and noncanonical NF- κ B pathways. In the canonical pathway, the prototypical NF- κ B, with a p65 (RELA)/p50 heterodimer, is kept inactive in the cytoplasm of quiescent cells by its inhibitor, I κ B α (27). Stimulation by proinflammatory cytokines or many other NF- κ B-inducing agents leads to the activation of the I κ B kinase (IKK) complex, consisting of kinase subunits IKK α and IKK β and the regulatory subunit IKK γ , or NEMO, which then phosphorylates I κ B α at two N-terminal serine residues to promote its polyubiquitination and degradation. Free NF- κ B then accumulates in the nucleus to activate target genes that play an important role in cell survival, cell proliferation, and the immune system (27). The noncanonical pathway is triggered by specific ligands, such as CD40 ligand/lymphotoxin β or BAFF, and is mediated through NIK-induced activation of IKK α , phosphorylation and processing of p100 to p52, and nuclear translocation of the p52/RelB complex for gene activation (12, 13, 14). In certain

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cell types, activation of NF- κ B by UV radiation can also occur via an IKK-independent pathway that involves casein kinase 2 (CK2)-mediated phosphorylation of I κ B α at its C terminus, followed by its degradation to liberate NF- κ B for nuclear entry and transcriptional gene activation (30).

Several oncogenic viruses, including Epstein-Barr virus (EBV), human herpesvirus 8 (HHV-8), and human T-cell leukemia virus 1 (HTLV-1), as well as HR mucosal HPV types, are able to activate NF- κ B, which in turn promotes cellular proliferation, immortalization, and transformation via upregulation of NF- κ B-responsive genes (5, 9, 20, 23, 25, 26, 31, 44).

In contrast to the mucosal HR HPV types, it is not known whether beta HPV types have the ability to target NF- κ B pathways or whether this event plays a role in beta HPV-mediated cellular immortalization and transformation.

In the present study, we show that E6 and E7 of HPV38 are potent activators of NF- κ B. Most importantly, this event appears to be important for protecting HPV38-immortalized keratinocytes against apoptosis induced by tumor necrosis factor alpha (TNF- α) and/or UV.

MATERIALS AND METHODS

Plasmid constructs. The retroviral expression of pLXSN-HPV38E6E7, pLXSN-HPV38E6, and pLXSN-HPV38E7 has been described previously (1, 10). pLXSN-HPV10E6, pLXSN-HPV10E7, pLXSN-HPV20E6, and pLXSN-HPV20E7 were generated by molecular cloning. The expression plasmid pcDNA3-CYLD was obtained from G. Mosialos (School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece). pcDNA3-myc-p100 was obtained from S. Ley (NIMR, London, United Kingdom). p-EGFP-C1 was obtained from BD Clontech (Palo Alto, CA). pcDNA3-LMP1 and anti-LMP1 antibody were obtained from E. Kieff (Harvard Medical School, Boston, MA). The expression retrovirus vector containing the NF- κ B superrepressor Δ N-I κ B α , which lacks the sequence that codes for the first 36 N-terminal amino acids (pBabe-puro- Δ N-I κ B α), was generated by introduction of the PCR-amplified DNA fragment from pcDNA3-Flag-I κ B α (obtained from T. Gilmore, Boston University, Boston, MA, and E. Kieff, Harvard Medical School, Boston, MA) into BamHI and EcoRI sites of pBabe-puro. The construct was verified by sequencing. Bay-11 was purchased from Calbiochem, San Diego, CA.

Cell culture and transfection. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (45). Primary human foreskin keratinocytes (HFks) or human foreskin keratinocytes immortalized by HPV38 E6 and E7 oncogenes (HEK 38 E6E7) were cultured as previously described (1, 10). Cell transfection was performed using the Fugene transfection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. In all cases, total DNA levels were normalized with empty vector. Small interfering RNA (siRNA) for p65 (siGenome Smart pool M-003533-02-0005), human RELA (NM_021975; Dharmacon, Lafayette, CO), or scrambled RNA used as a control (Ambion, Austin, TX) was transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Retroviral infection. The generation of high-titer retroviral stocks ($>5.0 \times 10^6$ IU/ml) by transfection of Phoenix cells, the infection of human keratinocytes, and the establishment of immortalized human keratinocytes were performed as previously described (1).

Cell proliferation assay. To assess cell proliferation, HEK 38 E6E7 and HEK 38 E6E7- Δ N-I κ B α cells were seeded at a density of 2×10^3 /cm² in 6-cm dishes. After 24 h, the cells were incubated with or without TNF- α (10 ng/ml) in combination or not with Bay11-7082 (1 μ M). The media were then changed every 48 h, and the cells were counted at regular intervals as indicated.

Luciferase reporter assay. Cells in six-well plates were transfected with NF- κ B-dependent firefly luciferase reporter construct (500 ng) together with a *Renilla* luciferase construct (50 ng). Forty-eight hours posttransfection, the cells were incubated with or without TNF- α (10 ng/ml) for 6 h. Whole-cell extracts were prepared, and the firefly, as well as the *Renilla*, luciferase activities were measured according to the protocol for the dual-luciferase system (Promega, Madison, WI).

TNF- α stimulation, UV light irradiation, and FACS analysis. Cells were incubated with TNF- α (10 ng/ml), and the stimulation was terminated by replac-

ing the medium with cold phosphate-buffered saline (PBS). UV irradiation was performed with UV-Bio-Spectra (Vilber Lourmat, Marne-La-Vallée, France). For irradiation, the medium was removed and replaced with PBS. The cells were irradiated with 80 mJ/cm² of UV radiation at 312 nm. After irradiation, fresh medium was added to the cells. To assess survival by fluorescence-activated cell sorting (FACS), cells were seeded in 6-cm dishes. After 24 h, they were irradiated with or without UV (312 nm; 80 mJ/cm²) or not irradiated and incubated with or without TNF- α (10 ng/ml). Cells were then collected, washed twice with PBS, and fixed with 70% ethanol on ice for 30 min. Approximately 10^6 cells were washed with PBS and resuspended in 500 μ l of PBS containing RNase A (0.1 mg/ml) and propidium iodide (5 μ g/ml). After incubation for 30 min on ice, the percentage of apoptotic cells was analyzed with a Becton Dickinson FACSort to determine the percentage of sub-G₀ cell population using the Cell Quest analysis software package.

Cell fractionation. Cytoplasmic and nuclear extracts were prepared using the Panomics nuclear extraction kit (Panomics, Inc., Fremont, CA) according to the manufacturer's instructions. Proteins in different cellular fractions were analyzed by immunoblotting.

Antibodies, immunoblotting, and immunofluorescence. Immunoblotting was performed as previously described (45). Briefly, cells were lysed in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 3% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM sodium fluoride, 1 mM sodium orthovanadate) for 30 min on ice. Insoluble material was removed by centrifugation at $14,000 \times g$ for 15 min. Whole-cell extracts were analyzed by immunoblotting. The antibodies used were as follows: p65, phospho-p65, phospho-I κ B α , p100, p52, PARP (Cell Signaling), β -tubulin, anti-Flag (Sigma), and actin (MP Biomedicals, Inc.). TNF- α was obtained from R&D Systems (Minneapolis, MN). For immunofluorescence, HEK 38 E6E7 and HEK 38 E6E7- Δ N-I κ B α cells were seeded on sterilized coverslips in 12-well plates at 1×10^5 cells per well. After exposure to TNF- α for the indicated periods, the cells were fixed with 4% paraformaldehyde for 10 min. The fixed cells were permeabilized with 0.1% Triton X-100 for 5 min and then blocked with 5% nonfat milk in 0.05% Tris-buffered saline-Tween at room temperature for 1 h, followed by incubation with the indicated primary antibodies and fluorescein isothiocyanate/tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies (Interchim) as described previously. The cellular localization of p65 was visualized using a fluorescence microscope, and random fields were photographed.

Reverse transcription (RT)-PCR and quantitative RT-PCR (qRT-PCR) analyses. Total RNA was extracted from cells with TRIzol reagent (Invitrogen), followed by DNase I treatment to prevent cellular DNA contamination in the PCR. cDNA was synthesized with the First Strand cDNA synthesis kit (MBI Fermentas, Heidelberg, Germany) with a random hexamer primer and Moloney murine leukemia virus reverse transcriptase. The primers used for detection of HPV38 E6 and E7, α IAP, α IAP1, α IAP2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described previously (10, 24). Real-time PCR was performed using the MesaGreen qPCR MasterMix Plus for SYBR Assay (Eurogentec, San Diego, CA). The following primers were used: α IAP, Fw (5'-TGGGGTTCAGTTTCAAGGAC-3') and Rev (5'-CGCCTTAGCTGCTCTTCAGT-3'); α IAP1, Fw (5'-CTGGGAACCAAAGGATGATG-3') and Rev (5'-ATTTCGAGCTGCATGTGTCTG-3'); α IAP2, Fw (5'-CCAAGTGGTTTCCAA GGTGT-3') and Rev (5'-TGGGCTGTCTGATGTGGATA-3'); HPV38 E6, Fw (5'-TCTGGACTCAAGAGGATTTTG-3') and Rev (5'-CACTTTAAACAATA CTGACACC-3'); HPV38 E7, Fw (5'-CAAGCTACTCTTCGTGATATAGTT C-3') and Rev (5'-CAGGTGGGACACAGAAGCCTTAC-3'); and GAPDH, Fw (5'-GCCAAAAGGGTCATCATC-3') and Rev (5'-TGCCAGTGAGCTTC CCGTTC-3'). GAPDH was used for normalization. Relative expression was calculated using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$) (34).

EMSA. Cells were collected and washed twice with PBS, and nuclear extracts were prepared as described previously (55). Briefly, cell pellets were resuspended in cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2 μ g/ml aprotinin, and 5% glycerol) and incubated on ice for 30 min with occasional mixing. Triton X-100 was then added to a final concentration of 0.2%, and the cell lysates were centrifuged at 5,000 rpm for 5 min to obtain nuclear pellets. The supernatants were collected as cytoplasmic fractions. The nuclear pellets were washed with buffer A, resuspended in buffer B (20 mM HEPES, pH 7.9, 450 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μ g/ml aprotinin, and 25% glycerol), and incubated on ice for 30 min with occasional mixing to extract nuclear proteins. Nuclear lysates were centrifuged at 14,000 rpm for 10 min, and the supernatants were collected as nuclear fractions. Protein was quantified using the Bradford assay. An electrophoretic mobility shift assay (EMSA) was performed using the EMSA Gel Shift kit (Panomics, Inc., Fremont,

CA) according to the manufacturer's instructions. Briefly, 5 μ g of total nuclear proteins was incubated at room temperature with biotin-labeled NF- κ B binding oligonucleotide, poly(dI-dC), and binding buffer. Complexes were resolved on a 5% nondenaturing acrylamide gel, followed by blotting on a nylon membrane and visualization by using avidin-labeled horseradish peroxidase (HRP) conjugate.

Colony formation assay. HFK 38 E6E7 and HFK 38 E6E7- Δ N1 κ B α cells were seeded at a density of 1×10^3 /6-cm dish. After 24 h, the cells were incubated with or without TNF- α (10 ng/ml). The medium was changed every 48 h, and the cells were grown for 8 to 10 days. After this period, the cells were washed with PBS, and colonies were fixed and stained on the plates with crystal violet in 20% methanol. The number of colonies was quantified in each plate by direct counting.

RESULTS

HPV38 E6 and E7 activate NF- κ B signaling. To determine whether HPV38 activates NF- κ B, HEK293 cells were transfected with HPV38 E6 or E7 expression constructs, together with a luciferase reporter with four upstream NF- κ B sites (49). As shown in Fig. 1A, both viral proteins, alone or in combination, induced κ B site reporter gene activity by about 5- and 7-fold, respectively, compared to the empty-vector control. To evaluate whether cutaneous HPVs, such as HPV10 and HPV20, which are not able to immortalize keratinocytes (10) are impaired in NF- κ B activation, HEK293 cells were transfected with HPV10 and HPV20 E6 and E7 expression plasmids, together with an NF- κ B-luciferase reporter. As shown in Fig. 1B and in agreement with previous observations, induction of NF- κ B was detected with HPV38 E6 and E7, but not with HPV10 and HPV20 oncoproteins. Thus, the capacity for HPV38 E6 and E7 to immortalize human primary keratinocytes correlates with their ability to activate NF- κ B.

Next, we assessed the effect of HPV38 E6 and E7 on activation of NF- κ B in human keratinocytes, which are the natural hosts of the virus. Primary HFKs were infected with empty retrovirus (pLXSN) or immortalized by transduction with a recombinant HPV38E6E7 retrovirus. RT-PCR analysis demonstrated the presence of mRNAs for both viral genes (Fig. 1C). A relative increase of phosphorylated I κ B α was also detected in HPV38E6E7-immortalized cells in comparison to primary cells transduced with an empty vector (Fig. 1D, with quantification on the right). This difference was more evident in HPV38E6E7 HFKs following treatment with TNF- α than in the control cells (Fig. 1D). To corroborate the initial data, the experiments were repeated with an additional cell line of HPV38E6E7 HFKs that was obtained using primary keratinocytes from another donor (Fig. 1E, with quantification on the right). In these immortalized keratinocytes, the constitutive levels of phosphorylated I κ B α were even higher than in the previously analyzed HPV38E6E7 HFKs (Fig. 1D and E). The effect of the oncoproteins on NF- κ B was further corroborated in an NF- κ B promoter reporter assay, which showed that an increase of NF- κ B activation occurred upon TNF- α treatment (Fig. 1F). The HPV38 E6- and E7-mediated activation of NF- κ B signaling was also confirmed in a third HPV38E6E7 HFK line that was obtained using primary cells from an additional donor (data not shown).

Since the phosphorylation and degradation of I κ B α depend on the canonical pathway of NF- κ B, we asked whether the noncanonical pathway can also be activated by HPV38 E6 and E7. To this end, we examined the effects of HPV38 E6 and E7

on the processing of p100/NF- κ B2 to p52, a hallmark of non-canonical NF- κ B activation (13, 14). Ectopic expression of EBV LMP1, a known inducer of both canonical and non-canonical NF- κ B (36), increased the processing of p100/NF- κ B2 to p52 (Fig. 1G). However, neither HPV38 E6 nor HPV38 E7 induced a significantly increased level of p52 (Fig. 1G). Thus, HPV38 E6 and E7 mainly induce the canonical pathway of NF- κ B activation.

To gain further insight into the mechanisms by which HPV38 E6 and E7 activate NF- κ B, we examined whether, like HPV16 E6 (3), HPV38 E6 and/or E7 can induce the degradation of the tumor suppressor CYLD, a deubiquitinating enzyme that inhibits NF- κ B activation (50). We found that neither HPV38 E6 nor E7 downregulates the levels of CYLD (Fig. 1H).

Taken together, these results show that HPV38 oncoproteins E6 and E7 activate the canonical pathway of NF- κ B and cooperate with TNF- α in NF- κ B activation through phosphorylation of I κ B α and p65.

Inhibition of NF- κ B signaling affects cellular proliferation and cell survival of HPV38E6E7 keratinocytes upon TNF- α treatment. Due to the importance of NF- κ B activity in cell survival, proliferation, and transformation, we next determined the effects of inhibition of NF- κ B signaling on these events in HPV38E6E7 HFKs. To inhibit the NF- κ B pathways, we used a deletion mutant of I κ B α (Δ N-I κ B α) that lacks the first N-terminal 36 amino acids (Fig. 2A) (7, 8). Δ N-I κ B α cannot be phosphorylated and degraded and thus blocks the activation of NF- κ B by retaining p65 in the cytoplasm (7). HPV38E6E7 HFKs were transduced with the empty retrovirus pBabe as a control or with the retrovirus expressing the superrepressor Δ N-I κ B α . As shown in Fig. 2B (left), Δ N-I κ B α was efficiently expressed in transduced cells, whereas no Δ N-I κ B α protein band was detected in the vector control-transduced cells. Δ N-I κ B α expression did not significantly alter the mRNA levels of HPV38 E6 and E7 (Fig. 2B, right, and C).

Exposure of cells to TNF- α did not affect the phosphorylation and protein levels of Δ N-I κ B α , in contrast to the endogenous wild-type I κ B α (Fig. 2D). Consistent with the data presented in Fig. 1A on I κ B α phosphorylation, partial localization of p65 in the nucleus was observed in unstimulated parental control HPV38E6E7-immortalized keratinocytes (Fig. 2E). This p65 nuclear accumulation gradually increased upon TNF- α treatment (Fig. 2E, left). In contrast, p65 was retained in the cytoplasm in HPV38E6E7 keratinocytes expressing Δ N-I κ B α following treatment with TNF- α (Fig. 2E, right). Similar results were obtained by cellular fractionation followed by p65 immunoblotting (Fig. 2F). To more directly determine the effect of expression of Δ N-I κ B α in keratinocytes on nuclear NF- κ B activity, electromobility shift and luciferase reporter assays were performed. As shown in Fig. 2G, TNF- α -induced nuclear κ B site binding activity was greatly decreased in Δ N-I κ B α HPV38E6E7 HFKs compared to control cells. Consistent with these results, Δ N-I κ B α significantly inhibited NF- κ B reporter gene activity in HPV38E6E7 keratinocytes (Fig. 2H).

We next examined whether inhibition of NF- κ B activity by Δ N-I κ B α affects the proliferation of HPV38E6E7 HFKs. Under normal growth conditions, Δ N-I κ B α expression did not significantly affect the proliferation rate of HPV38E6E7 HFKs in comparison to the control cells (Fig. 3A). Exposure to

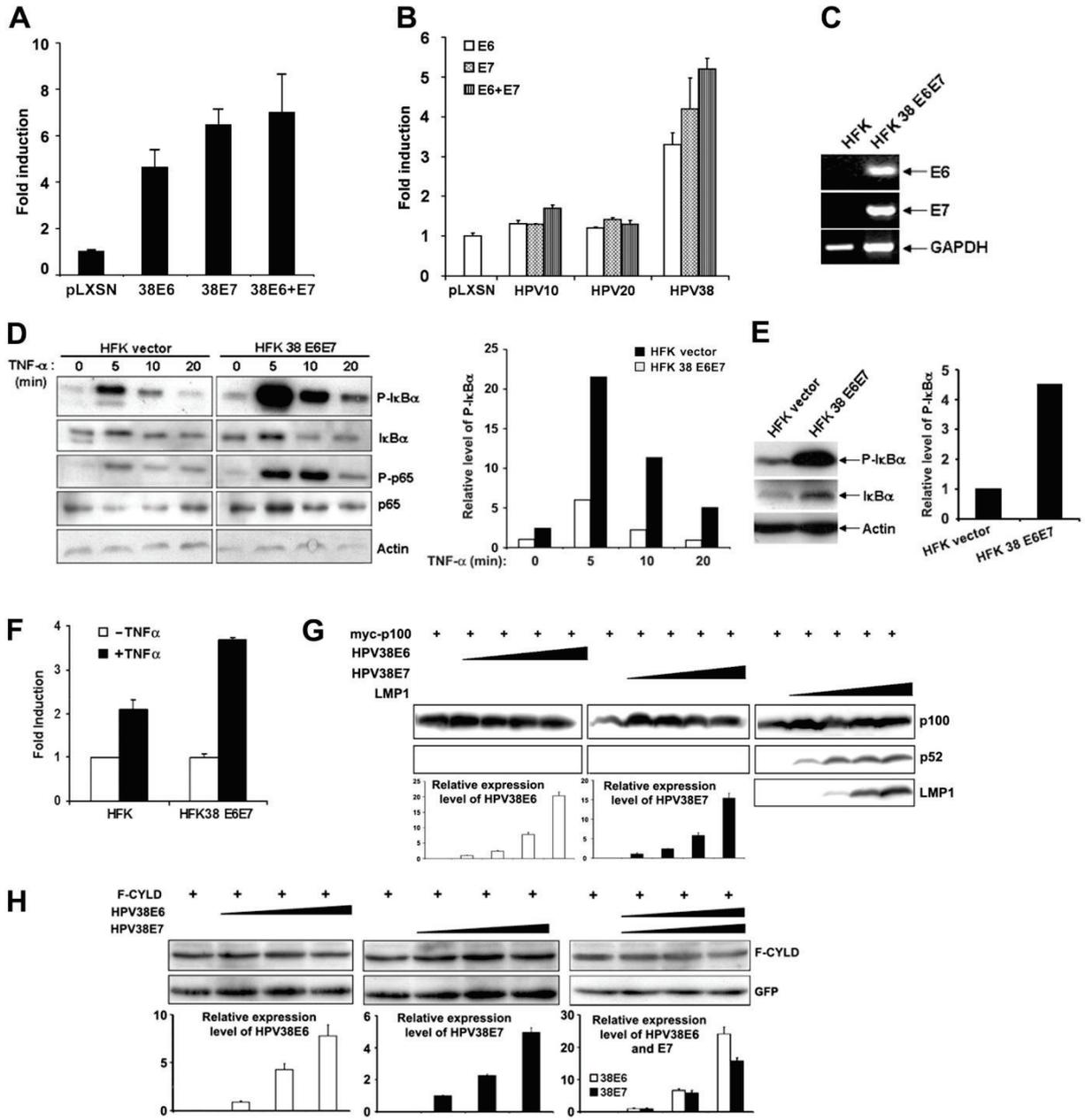


FIG. 1. HPV38 E6 and E7 activate NF-κB and sensitize HFKs to TNF-α-mediated NF-κB activation. (A) HEK293 cells were transiently transfected with an NF-κB-dependent firefly luciferase reporter construct (500 ng), together with a *Renilla* luciferase construct (50 ng), with or without the different expression vectors for HPV38 E6 and HPV38 E7 oncogenes. The cells were harvested 48 h posttransfection, whole-cell extracts were prepared, and the firefly and *Renilla* luciferase activities were measured. The fold induction of NF-κB activity was determined. The experiment was performed at least three times independently in duplicate. The error bars indicate standard deviations. (B) HEK293 cells were transiently transfected with an NF-κB-dependent luciferase reporter construct, together with a *Renilla* luciferase construct with or without the different pLXSN expression vectors for HPV10 E6, HPV10 E7, HPV20 E6, HPV20 E7, HPV38 E6, and HPV38 E7 oncogenes. The *Renilla* luciferase activity was measured, and the fold induction of NF-κB activity was determined. The data are representative of at least three independent experiments performed in duplicate. (C) Total RNA was isolated from HFKs transfected with the empty vector pBabe (HFK) and immortalized HFKs expressing HPV38E6E7 (HFK 38 E6E7) using TRIzol reagent, and cDNA was prepared by RT-PCR. The cDNA obtained was used as a template for determination of the expression of HPV38 E6 and E7 genes with GAPDH expression as a control. (D) HFKs and HFKs immortalized by HPV38E6E7 were incubated with or without TNF-α (10 ng/ml) for the indicated periods, and whole-cell extracts were analyzed by immunoblotting with actin as a loading control. Quantification of the level of phosphorylated IκBα (P-IκBα) relative to time zero is presented (right). (E) HFKs containing empty vector and immortalized HFK 38 E6E7 established from keratinocytes from a different donor than in panel

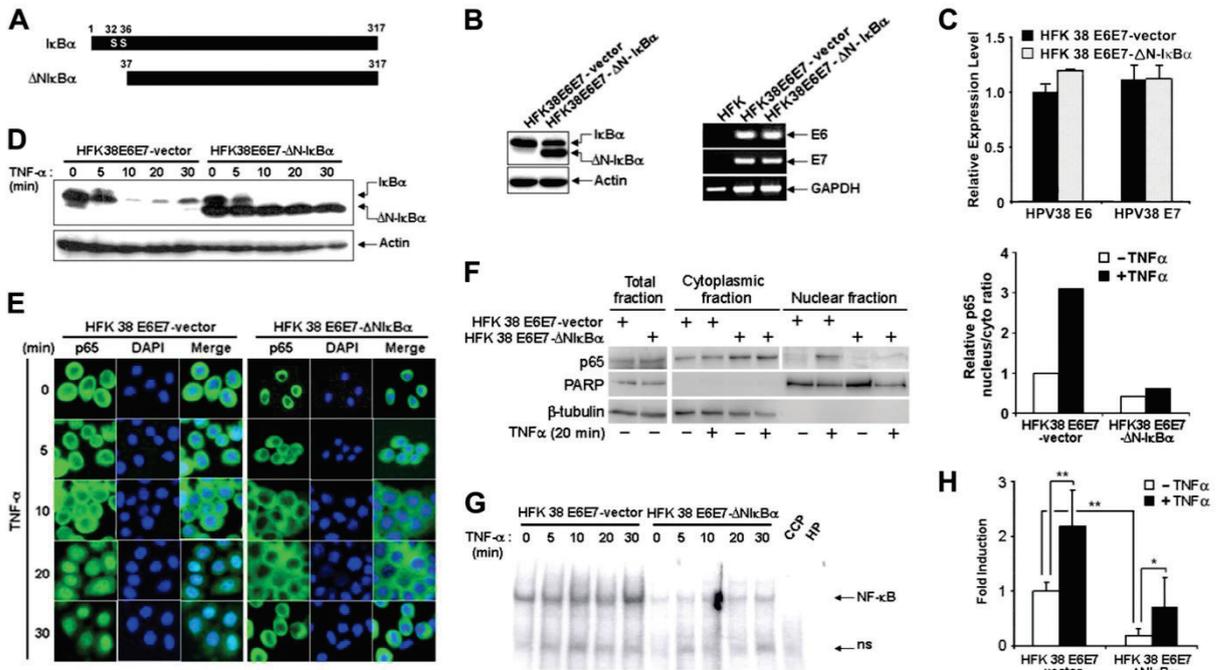


FIG. 2. Nuclear translocation and activity of NF- κ B (p65) induced by TNF- α is abrogated in HFK 38 E6E7 expressing the I κ B α superrepressor Δ N1 κ B α . (A) Schematic representation of the generation of Δ N-I κ B α by PCR. (B) Whole-cell extracts were prepared from HFK 38 E6E7-containing vector and HFK 38 E6E7 expressing Δ N-I κ B α (HFK38E6E7- Δ N-I κ B α) and analyzed by immunoblotting for I κ B α with actin as a loading control (left). Total RNA was isolated from HFKs, HFK 38 E6E7, and HFK 38 E6E7- Δ N-I κ B α using TRIzol reagent, and cDNA was prepared by RT-PCR. The cDNA obtained was used as a template for determination of the expression of HPV38 E6 and E7 genes with GAPDH expression as a control (right). (C) The expression levels of HPV38 E6 and E7 in HFK 38 E6E7-vector and in HFK 38 E6E7- Δ N-I κ B α were determined by qRT-PCR. The error bars indicate standard deviations. (D) HFK 38 E6E7 and HFK 38 E6E7- Δ N-I κ B α were incubated with or without TNF- α (10 ng/ml) for the indicated periods, and whole-cell extracts were analyzed by immunoblotting for I κ B α with actin as a loading control. (E) HFK 38 E6E7 and HFK 38 E6E7- Δ N-I κ B α were incubated with or without TNF- α (10 ng/ml) for the indicated periods and stained for NF- κ B (p65) and nucleus (DAPI [4',6-diamidino-2-phenylindole]). (F) HFK 38 E6E7-vector and HFK 38 E6E7- Δ N-I κ B α were treated or not with TNF- α (10 ng/ml) for 20 min. Cellular cytoplasmic and nuclear fractions, as well as total lysate, were then prepared and analyzed by immunoblotting. β -Tubulin and PARP were used as controls for the cellular fractionation (left). Quantification of the relative nuclear p65 fraction is also shown (right). (G) HFK 38 E6E7 and HFK 38 E6E7- Δ N-I κ B α were incubated with TNF- α (10 ng/ml) for the indicated periods, and nuclear proteins were extracted and incubated with labeled or unlabeled (CCP) NF- κ B-binding oligonucleotides. The DNA-protein complexes were separated on a polyacrylamide gel, followed by blotting and detection with an EMSA Gel shift kit (Panominc Inc.). (H) HFK 38 E6E7 and HFK 38 E6E7- Δ N-I κ B α were transiently transfected with an NF- κ B-dependent firefly luciferase reporter construct, together with a *Renilla* luciferase construct. Forty-eight hours posttransfection, the cells were incubated with or without TNF- α (10 ng/ml) for 6 h. The firefly and *Renilla* luciferase activities and the NF- κ B fold induction were then determined. The experiments were performed at least three times independently in duplicate. ** and *, $P < 0.01$ and $P < 0.05$, respectively.

TNF- α resulted in significant inhibition of proliferation of both control cells and cells expressing the I κ B α superrepressor. However, the proliferation of HPV38E6E7-immortalized keratinocytes with impaired NF- κ B was substantially more inhibited than that of the control cells (Fig. 3A). Additional exper-

iments showed that cells expressing the I κ B α superrepressor formed 4 to 10 times fewer colonies than the parental HPV38E6E7-immortalized keratinocytes following exposure to TNF- α (Fig. 3B and C). Next, we determined whether inhibition of the NF- κ B complex by an alternative method could

D were analyzed by immunoblotting for the expression of P-I κ B α , with actin as a loading control. On the right is shown the quantification of the relative levels of P-I κ B α . (F) HFKs and HPV38E6E7 HFKs (HFK38 E6E7) were transiently transfected with a NF- κ B-dependent firefly luciferase reporter construct, and the luciferase activity and NF- κ B fold induction were determined as in panel A. The experiment was performed at least three times independently in duplicate. (G) HEK293 cells were transiently transfected with a constant amount of myc-p100 expression plasmid with increased concentrations of expression vectors for HPV38 E6, HPV38 E7, or EBV LMP1. The expression of p100, p52, and LMP1 was monitored by immunoblotting. The expression levels of HPV38 E6 and HPV38 E7 were determined by qRT-PCR (bottom). (H) HEK293 cells were transiently transfected with a constant amount of F-CYLD expression plasmid with increased concentrations of expression vectors for HPV38 E6, HPV38 E7, or both together. F-CYLD protein was revealed by immunoblotting using anti-Flag antibodies. The expression levels of HPV38 E6 and HPV38 E7 were determined by qRT-PCR as in panel F (bottom). Green fluorescent protein (GFP) was used as an internal control for transfection and loading.

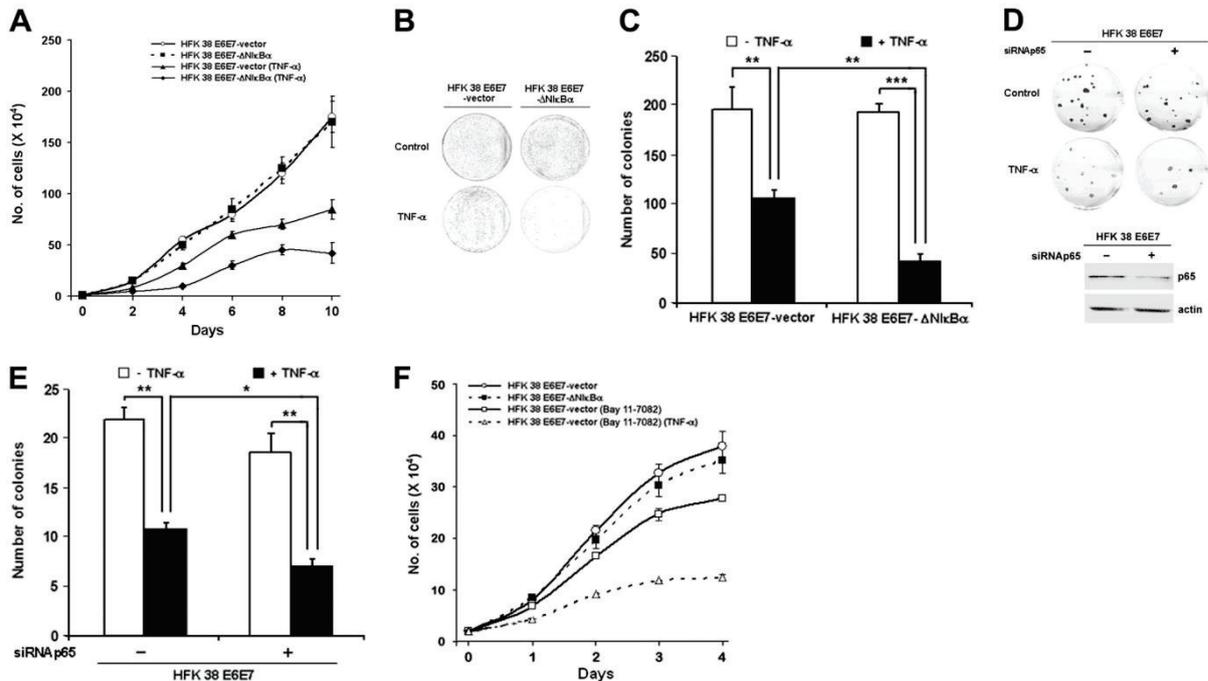


FIG. 3. NF- κ B contributes to the proliferation and survival of HFK 38 E6E7. (A) HFK 38 E6E7 and HFK 38 E6E7- Δ N- $\text{I}\kappa$ B α were exposed or not exposed to TNF- α (10 ng/ml), and the cells were counted at regular intervals as indicated. The experiments were performed at least three times independently in duplicate. The error bars indicate standard deviations. (B) HFK 38 E6E7 and HFK 38 E6E7- Δ N- $\text{I}\kappa$ B α were exposed or not to TNF- α as indicated, followed by a colony formation assay. (C) Quantification of the colony formation assay presented in panel B. ** and ***, $P < 0.01$ and $P < 0.001$, respectively. (D) HFK 38 E6E7 transfected with siRNA control or siRNA for p65 was exposed or not to TNF- α , followed by a colony formation assay (top). The expression level of p65 is shown below. (E) Quantification of the colony formation assay in panel D. *, $P < 0.05$. (F) Cells were treated with the chemical inhibitor of NF- κ B, Bay-11, with or without TNF- α and processed as in panel A.

result in the same phenomenon. Indeed, p65 gene silencing by siRNAp65 in HPV38E6E7-immortalized keratinocytes led to a significant decrease in colony formation upon TNF- α exposure (Fig. 3D and E). To further corroborate these data, cells were exposed to the chemical NF- κ B signaling pathway inhibitor Bay-11, which blocks the activation of IKK complex. Bay-11 significantly reduced the proliferation of HPV38E6E7-immortalized-keratinocytes in comparison to the control untreated cells (Fig. 3F) as a result of NF- κ B inhibition. As observed for Δ N- $\text{I}\kappa$ B α , the inhibition of proliferation is enhanced in Bay-11-treated cells exposed to TNF- α (Fig. 3F). The inhibition of proliferation observed in cells treated with Bay-11 in the absence of TNF- α contrasts with what was observed in cells where NF- κ B was compromised with Δ N- $\text{I}\kappa$ B α (Fig. 1A). This difference may be due to the key role of IKK β in promoting accumulation of the p53 antagonist Δ Np73 α , a function that is independent of NF- κ B activation (2). However, the fact that Bay-11 treatment strongly enhanced the inhibition of cellular proliferation induced by TNF- α fully confirmed our initial data obtained in Δ N- $\text{I}\kappa$ B α -expressing cells. Thus, HPV38 E6 and E7 attenuate the TNF- α -induced inhibition of cellular proliferation via activation of NF- κ B signaling.

HPV38 E6 and E7 inhibit TNF- α -induced apoptosis by increasing the expression of NF- κ B-regulated survival factor genes. We next sought to determine whether the inhibition of NF- κ B signaling in HPV38E6E7 HFKs influences the apopto-

sis induced by TNF- α and/or UV irradiation. The latter treatment was included in our experiments because it is known to be a strong inducer of TNF- α production and a key risk factor in skin carcinogenesis.

To evaluate the apoptotic response induced by TNF- α or UV irradiation in HPV38E6E7 HFKs, we first analyzed the cleavage of the caspase substrate PARP, which is an event closely associated with apoptosis. As observed previously in a different study on HPV16 (4), TNF- α treatment of HPV38E6E7 HFKs induced a low level of apoptosis with cleavage of a small proportion of PARP, while this event was strongly enhanced in cells expressing Δ N- $\text{I}\kappa$ B α (Fig. 4A, left). Similarly, PARP cleavage induced by UV irradiation alone or in combination with TNF- α was more evident in Δ N- $\text{I}\kappa$ B α HPV38E6E7 than in control cells (Fig. 4A, center and left). Quantification of the data clearly shows that more cleaved PARP was observed in cells with compromised NF- κ B signaling than in control cells (Fig. 4A, bottom). In agreement with the PARP cleavage data, FACS analysis showed that upon TNF- α or UV exposure, the percentage of apoptotic cells with a sub- G_0 content was higher in HPV38E6E7 HFKs with inhibited NF- κ B signaling (Fig. 4B).

NF- κ B activity protects cells against apoptosis by inducing the expression of a variety of cell survival genes (29, 54). Therefore, we sought to determine whether the increased apoptosis observed upon TNF- α or UV exposure is due to

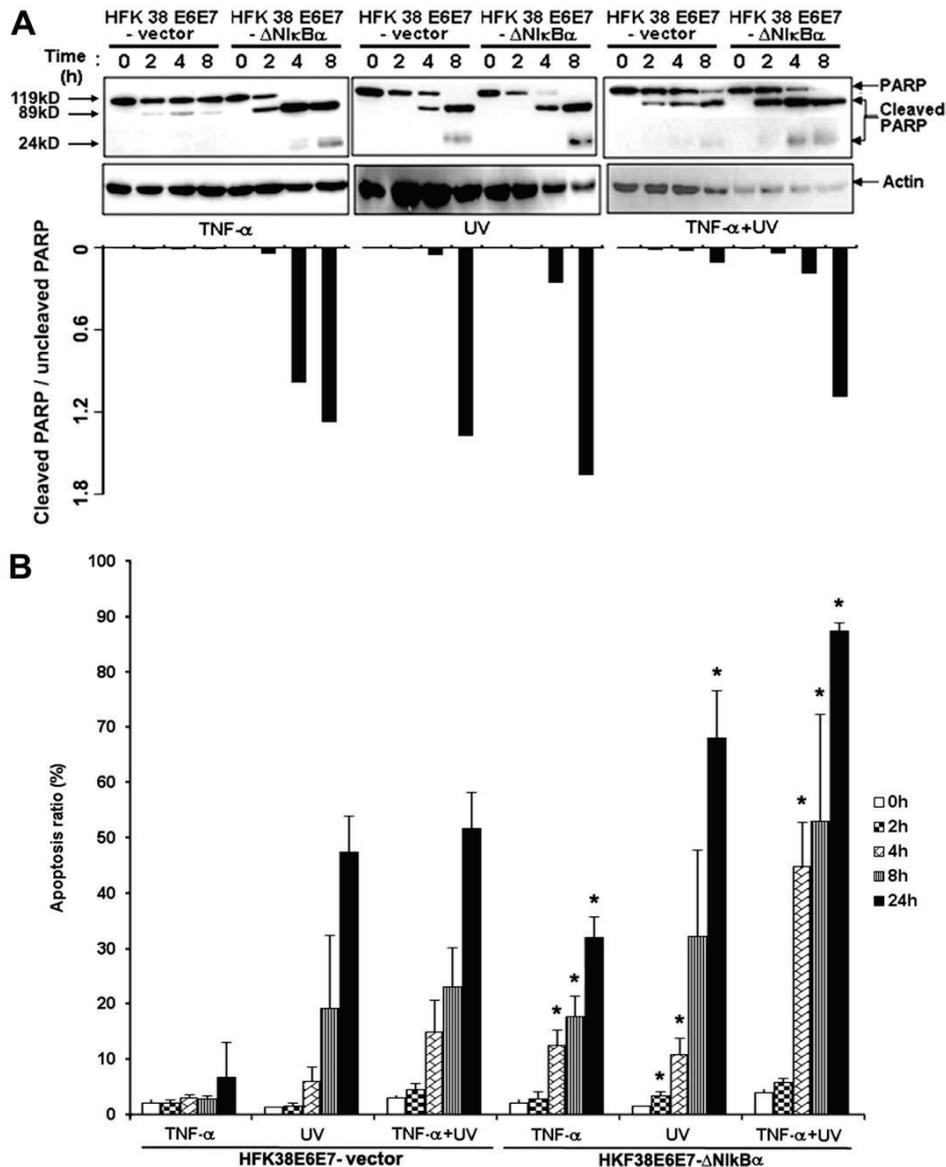


FIG. 4. NF- κ B inhibition induces apoptosis in immortalized HFK 38 E6E7. (A) (Top) HFK 38 E6E7 and HFK 38 E6E7- Δ N κ B α were incubated with TNF- α (10 ng/ml) or irradiated with UVB (312 nm; 80 mJ/cm²) or both for the indicated periods, and the whole-cell extracts were analyzed by immunoblotting for PARP with actin as a loading control. (Bottom) Quantification of relative cleaved PARP product obtained as shown above. (B) HFK 38 E6E7 and HFK 38 E6E7- Δ N κ B α were incubated with TNF- α (10 ng/ml) or irradiated with UVB (312 nm; 80 mJ/cm²) for the indicated periods. Subsequently, the cells were fixed and stained with propidium iodide, and the percentage of sub-G₀ cell population was detected by FACS. The experiments were performed at least three times independently in duplicate. An asterisk indicates that a statistical difference exists between HFK 38 E6E7- Δ N κ B α and HFK 38 E6E7-vector at that time point with a *P* value of <0.05. The error bars indicate standard deviations.

altered expression of three survival factors, cIAP, cIAP2, and xIAP, which are encoded by NF- κ B-regulated genes (22). While TNF- α exposure, as expected, did increase the level of mRNA expression of cIAP1, cIAP2, and xIAP in the control cells, a substantial reduction of the expression of cIAP2 and xIAP was observed in Δ N- κ B α HPV38E6E7 HFKs and to a lesser extent for cIAP1 (Fig. 5). Interestingly,

inhibition of the expression of HPV38 E6 and E7 by using siRNA resulted in apoptosis (Accardi et al., unpublished), likely a result of inhibition of the expression of survival factors.

Altogether, these results show that HPV38-induced NF- κ B signaling activation is important for immortalization and that this event plays a key role in favoring the survival of keratino-

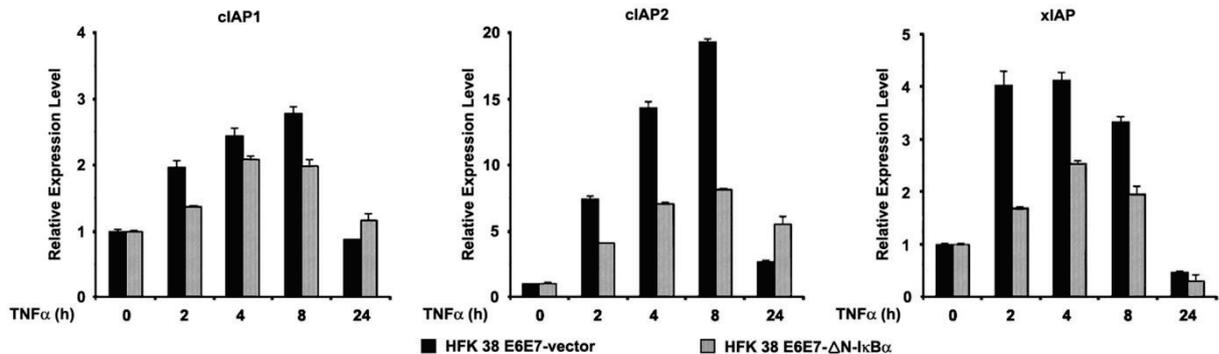


FIG. 5. NF- κ B-regulated anti-apoptotic genes are important for the survival of HFK 38 E6E7. HFK 38 E6E7 and HFK 38 E6E7- Δ N-I κ B α were incubated with TNF- α (10 ng/ml) for the indicated periods, and the total RNA extracted was analyzed by qRT-PCR for expression of cIAP1, cIAP2, and XIAP. The data are representative of three independent experiments with a P value of <0.05 . The error bars indicate standard deviations.

cytes upon TNF- α or UV exposure via expression of NF- κ B-regulated survival factor genes.

DISCUSSION

We have previously shown that E6 and E7 from beta cutaneous HPV38 displayed transforming activities in *in vitro* and *in vivo* experimental models (1, 17). Here, we report that HPV38 E6 and E7, similar to HPV16 E6 (28) and to the oncoprotein LMP1 of EBV (8, 49), are able to induce activation of NF- κ B in human keratinocytes. Both HPV38 E6 and E7 can independently activate NF- κ B (Fig. 1A). However, no significant increase in NF- κ B activation was observed when the two oncoproteins were expressed together (Fig. 1A), probably as a result of a saturation effect. In addition, NF- κ B activation upon TNF- α exposure was strongly increased in HPV38E6E7-immortalized keratinocytes in comparison to primary cells (Fig. 1D). Most importantly, we show that inhibition of NF- κ B signaling by different means sensitizes HPV38E6E7-immortalized keratinocytes to TNF- α - and UV-induced apoptosis. This event is associated with downregulation of the expression of survival factors regulated by NF- κ B. Interestingly, E6 and E7 from cutaneous HPVs that cannot promote keratinocyte immortalization are impaired in inducing NF- κ B activation (Fig. 1B), indicating a link between NF- κ B activation and E6- and E7-mediated cell immortalization.

It was previously shown that HPV16E6E7 keratinocytes are protected from apoptosis through NF- κ B activation (11, 28, 32, 44). Interestingly, cIAP-2, the main survival factor of human keratinocytes that is induced by HPV16 E6 through NF- κ B activation (28, 56), is also significantly upregulated in HPV38E6E7-immortalized keratinocytes in an NF- κ B-dependent manner. Thus, the two HPV types show some similarity in their molecular mechanisms. However, the ability to activate NF- κ B appeared not to be shared with E6 and E7 from cutaneous HPV types that display weak *in vitro* transforming activities (Fig. 1B), suggesting that this function may be linked to cellular transformation.

The mechanisms by which HPV38 E6 and E7 activate NF- κ B remain unclear. However, it is likely that E6 and E7 from HPV38 act at different levels of the NF- κ B signaling

pathway. It has been demonstrated that the PDZ binding motif present in HPV16 E6 is important for HPV16 E6-induced NF- κ B activation (28). Activation of NF- κ B by HPV16 E6 is also associated with the degradation of the deubiquitinating enzyme CYLD, an inhibitor of NF- κ B signaling (3). The absence of a PDZ binding motif in HPV38 E6 and in the E6s of other cutaneous HPVs and the inability of HPV38 E6 and E7 to induce CYLD degradation (Fig. 1H) suggest that HPV38 E6 and E7 activate NF- κ B by mechanisms different from those of HPV16 E6. Our data also reveal that, in contrast to another viral oncoprotein, LMP1 (36), the canonical pathway appears to be the major pathway used by HPV38 E6 and E7 to activate NF- κ B.

NF- κ B has been found to be an important modulator of the expression of various genes contributing to the malignant phenotype in human squamous cell carcinoma (SCC) (35). In addition, NF- κ B is constitutively active in murine and human SCC (18, 19, 53), and inhibition of NF- κ B in these cancer cells abrogates cell survival and tumor growth (19, 39). However, several studies have reported that transgenic mice expressing a nondegradable form of I κ B α show increased susceptibility to developing squamous cell carcinoma following increased apoptosis (52). Inactivation of IKK α increases skin tumor formation (15, 46, 57). In addition, decreased expression of IKK α was also observed in human skin and lung SCC (33, 46, 53). This discrepancy can result in differences in the cell system environment and the nature of targeted cells (54). In virus-induced carcinogenesis, NF- κ B might be required at the earliest stage of carcinogenesis of skin keratinocytes. However, inhibiting NF- κ B in keratinocytes immortalized by HPV38E6E7 did not significantly affect the growth of cells in comparison to control cells (Fig. 3). It is possible that in this particular context, the lack of a requirement for NF- κ B for cell growth may result from additional cellular events, such as activation of hTERT and chromosome rearrangements (21). Alternatively, HPV38E6E7 may activate a parallel cellular signaling pathway, such as AP-1, that has a partial protective effect, as demonstrated for HPV16E6E7 (32). This situation contrasts with the case, for example, of immortalization of primary B cells by EBV, which requires NF- κ B activation. Here, block-

ing NF- κ B spontaneously induced apoptosis in the EBV-immortalized lymphoblastoid cell lines (9).

In conclusion, our data show that NF- κ B activation in HPV38 E6 and E7 keratinocytes strongly alleviates the anti-proliferative response of UV and/or the proinflammatory cytokine TNF- α and that a correlation exists between NF- κ B activation and the ability of cutaneous HPV oncoproteins to promote keratinocyte immortalization. Since UV irradiation is considered a key risk factor in skin carcinogenesis, our findings provide evidence for cooperation between UV and beta HPV infection in promoting skin cancer.

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Chapter II: Results 3

**Supplementary results: Human papillomavirus type 38 protein E7 induces
NF- κ B pathway and interacts with IKK β**

1/ HPV38 E6 and E7 oncoproteins expression directly influence NF- κ B transcriptional activity

To determine the impact of HPV38 E6 and E7 expression on the NF- κ B pathway, we have analyzed the status of key players of this pathway in primary keratinocytes transduced with an empty retrovirus or with recombinant retroviruses expressing E6 and E7 from HPV10, 14 or 38. E6 and E7 from HPV10 and HPV14 do not display any *in vitro* transforming activities, while HPV38 E6 and E7 are able to efficiently immortalize primary human foreskin keratinocytes (38E6E7 HFK). The levels of phosphorylated I κ B α (serine 32 and 36) and p65 (serine 536) were determined by immunoblotting in primary cells or cells expressing E6 and E7 from three different HPV types. As shown in Figure 1A, higher levels of phosphorylated I κ B α and p65 were detected in 38E6E7 HFK in comparison to primary keratinocytes or keratinocytes expressing HPV10 or HPV14 E6 and E7. To corroborate these findings, we investigated whether HPV38 oncoproteins have the ability to modulate IKK complex activity. To this end, 38E6E7 HFK constitutively expressing a pRetrosuper (pRS) containing shRNA for HPV38 E6 and E7 were generated. Efficiency of E6 and E7 expression inhibition was measured by quantitative PCR (Figure 1D). IKK β was then isolated by immunoprecipitation from HPV38 pRS38E6/E7 and from HPV38 pRS keratinocytes and incubated with GST-I κ B α substrate in presence of [γ -³²P]ATP. The *in vitro* kinase assay showed a higher kinase activity of IKK β in presence of the viral proteins supporting the previous result (Figure 1B). Consistently, in the same cellular system, we observed a reduction in the protein levels of phospho-I κ B α and phospho-p65 in presence of the shRNA for HPV38 E6 and E7 (Figure 1C). Finally, we verified the transcriptional activity of NF- κ B in the same cells by measuring the mRNA levels of xIAP, cIAP1 and cIAP2 transcripts, products of NF- κ B anti-apoptotic target genes. As expected, we observed lower expression levels of xIAP, cIAP1 and cIAP2 genes in the pRS38E6/E7 cells compared to the cells harboring the pRS empty vector (Figure 1D). These results show that NF- κ B pathway is up-regulated in 38E6E7 HFK through the induction of the kinase activity of IKK β .

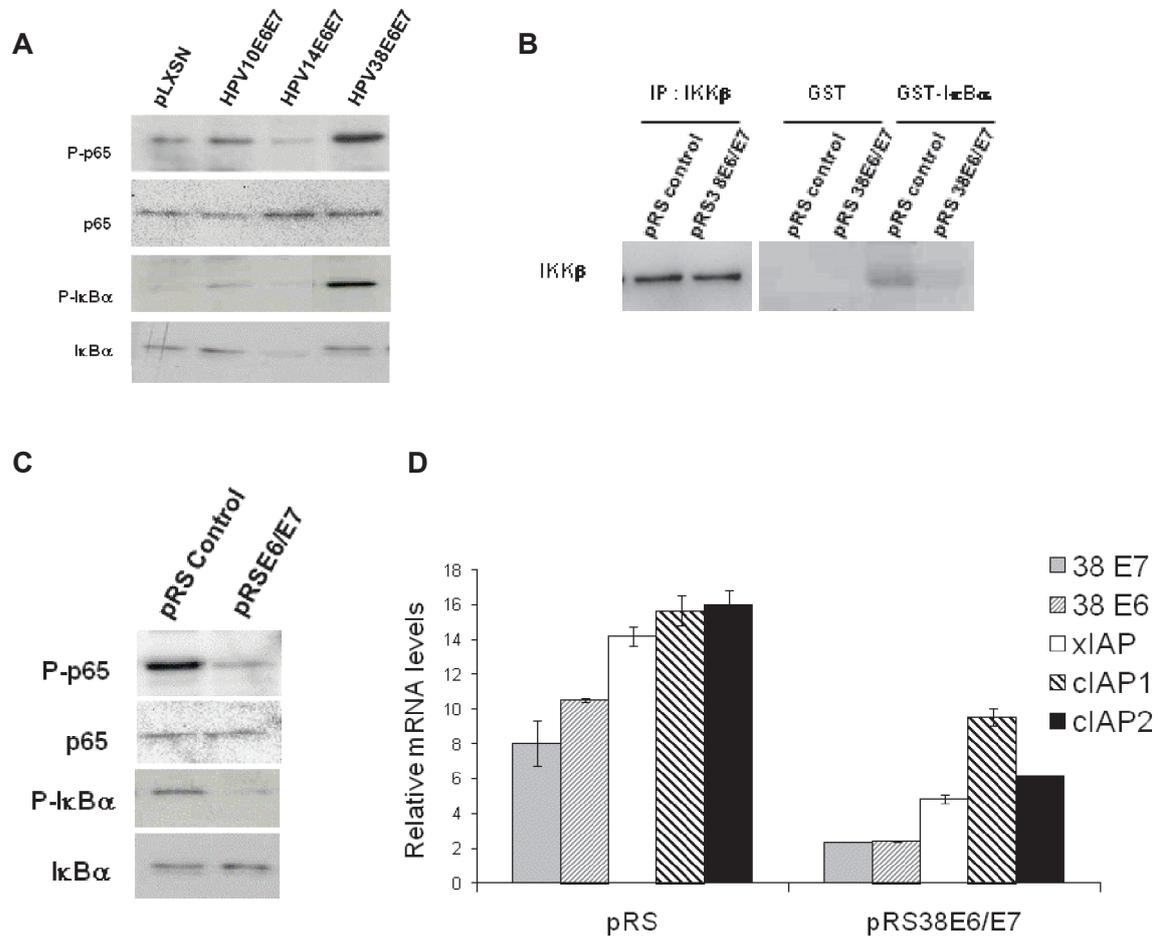


Figure 1. HPV38 E6 and E7 influence directly IKK β and NF- κ B activities

A/ Immunoblot on total proteins from cellular lysates of HFKs expressing E6 and E7 oncoproteins from HPV10, 14 or 38 or transfected with the pLXSN empty vector. Antibodies were used to look for the expression of phosphorylated-I κ B α and phosphorylated-p65 compared to the total I κ B α and p65 protein amount. B/ *In vitro* kinase assay using same amounts of immunoprecipitated IKK β proteins from 38E6/E7HFKs expressing pRS38E6/E7 or with the empty pRS vector (left panel). IKK β proteins were incubated with GST or GST-I κ B α during 30min at 30°C in presence of [γ - 32 P]ATP and radio activity is then detected. C/ HPV38 E6/E7HFKs expressing pRS38E6/E7 or pRS empty were used to detect by immunoblotting the protein levels of phospho-I κ B α and phospho-p65 compared to the total amounts of this proteins. D/ Quantitative PCR on the total cDNA obtained with RT-PCR on total RNA isolated from 38E6/E7HFKs expressing pRS38E6/E7 or containing the empty pRS vector. The expression levels of HPV38E6, HPV38E7, xIAP, cIAP1 and cIAP2 were analysed and normalized on the GAPDH expression level.

2/ E7 oncoprotein plays an important role in HPV38-mediated up-regulation of NF- κ B pathway

Next, we asked whether the activation of IKK activity was due to the expression of E6 and/or E7. Therefore, we transduced HFK with recombinant retroviruses expressing HPV38 E6 or E7. The levels of phosphorylated I κ B α and IKK α/β were determined in these cells by immunoblotting, since phosphorylation of IKK α at serine 176 and IKK β at serine 188 are hallmark of activation of the IKK complex. Higher amounts of phospho-I κ B α and phospho-IKK α/β were observed in the keratinocytes expressing 38E7 than in the one expressing 38E6, supporting a major role of HPV38 E7 in the events described so far (Figure 2).

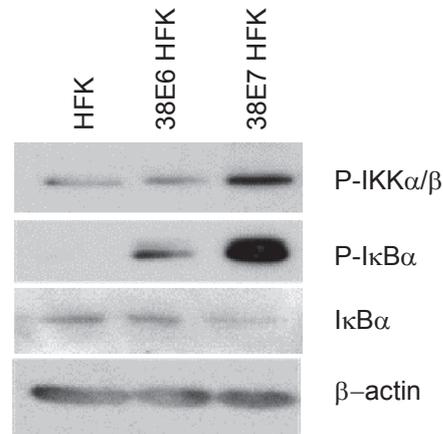


Figure 2. NF- κ B pathway activation in primary keratinocytes expressing HPV38E6 or HPV38E7

Primary keratinocytes expressing individually the oncoproteins HPV38E6 and HPV38E7 or with a pLXSN empty vector were collected at early passages for immunoblotting assay. Protein levels of phosphorylated IKK α/β and phosphorylated I κ B α were detected and compared to the total I κ B α amounts and β -actin.

3/ IKK β physically interacts with HPV38 E7 and is translocated into the nucleus

It has been previously shown that certain viruses constitutively activate NF- κ B using different strategies. HTLV-1 Tax oncoprotein was shown to interact with the IKK complex by direct binding of NEMO (IKK γ). Therefore, we asked whether similarly to Tax protein, HPV38 E7 could target the IKK complex. To explore this hypothesis, we first investigated IKK α and IKK β sub-cellular localization performing immunofluorescence staining on 38E6E7 HFK. Previous findings have shown that IKK β is localized in the cytoplasm, while IKK α is also cytoplasmic but with a shuttling capacity between the cytoplasm and the nucleus. In addition, studies on E7 from different HPV types indicate that the viral protein can shuttle between the nucleus and cytoplasm. We analyzed IKK α and IKK β localizations in 38E6E7 HFK, and the result shows that in contrast to IKK α , which appeared cytoplasmic, IKK β was localized in the nucleus (Figure 3A). In agreement with our previous results (Chapter I, Results I), we found that IKK β

nuclear localization was associated with HPV38 E7 expression. Indeed, HPV38 E6 expressing HFK showed a similar IKK β cellular localization to primary HFK, (Chapter II, Results 1). Since HPV38 E7 and IKK β appeared to co-localize we asked whether they physically interact. We therefore transiently transfected Human Embryonic Kidney (HEK) 293 cells with Flag-38E7 and un-tagged IKK β , and cellular extracts were prepared for immunoprecipitation of Flag-38E7 or IKK β . The immunoblotting analysis shows that the two proteins co-immunoprecipitated, indicating a physical interaction between HPV38 E7 and IKK β (Figure 3B). This result correlates with the IKK β translocation in the nucleus in presence of HPV38 E7 protein.

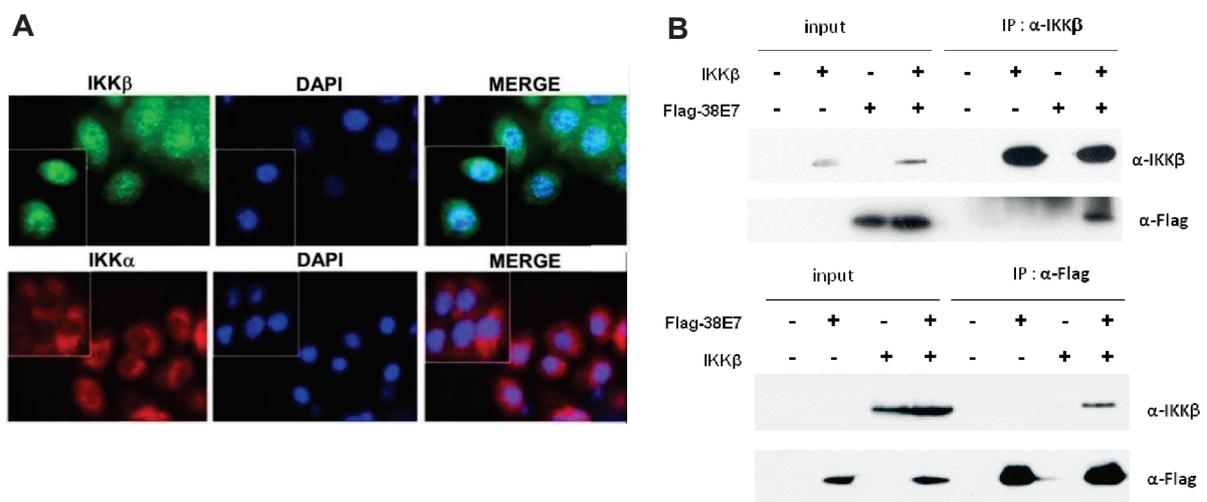


Figure 3. IKK β interacts with HPV38 E7 and accumulated in the nucleus

A/ Co-immunoprecipitation analysis using cell lysates from 293 cells transiently transfected with IKK β Flag-38E7. Anti-IKK β and anti-Flag antibodies were used for the immunoprecipitation and for immunoblotting.
 B/ Immunofluorescence staining of IKK β and IKK α in human foreskin keratinocytes expressing HPV38E6 and E7 together. DAPI is used to detect the nuclei.

4/ IKK β is localized in the nucleus of several cancer cell lines

The studies on HPV38 E6 and E7 HFK highlighted a key role of IKK β nuclear localization in cellular proliferation. To determine the role of IKK β in additional transformed cells, we

explored the localization of IKK β in cancer cell lines of different origins. Head and neck

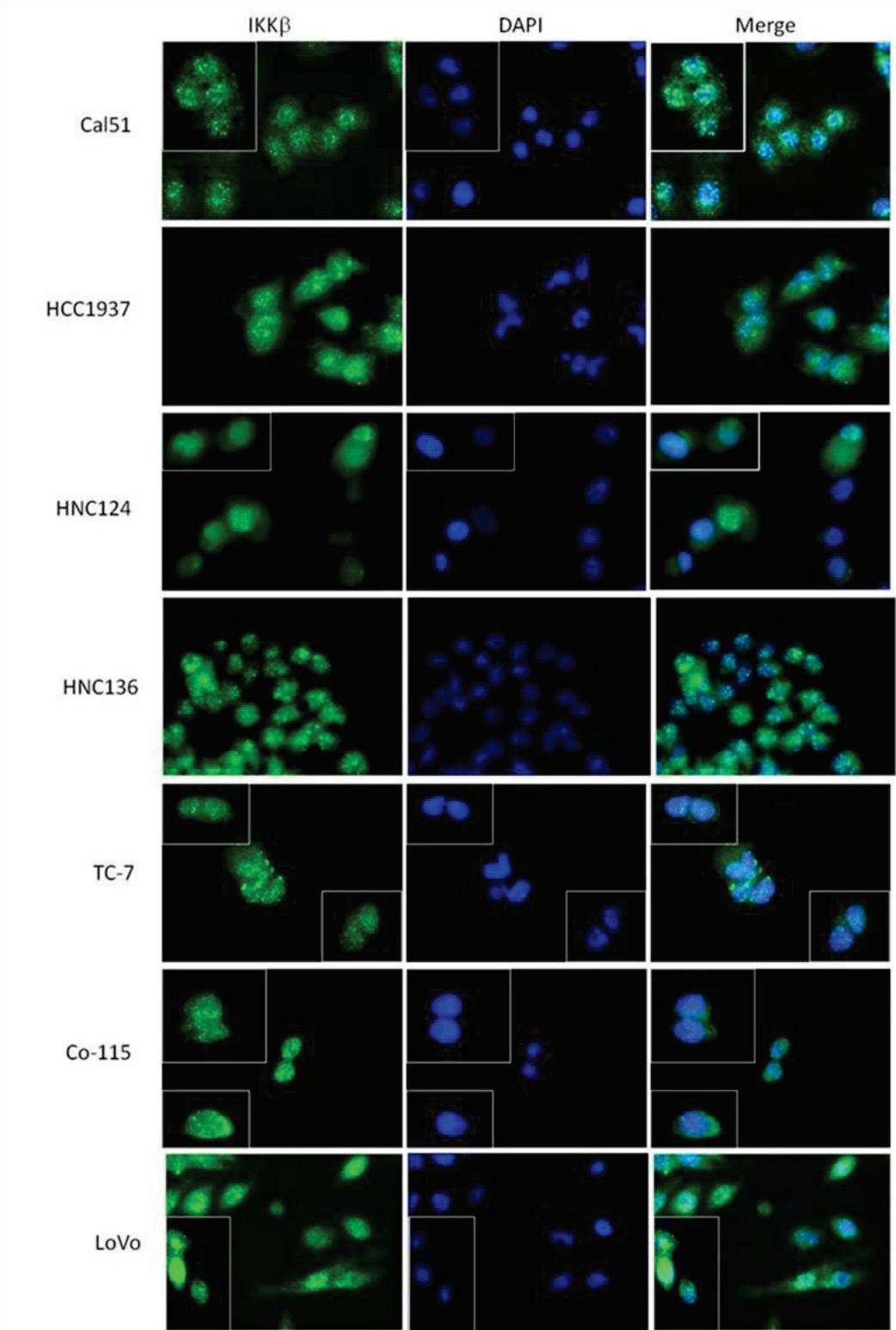
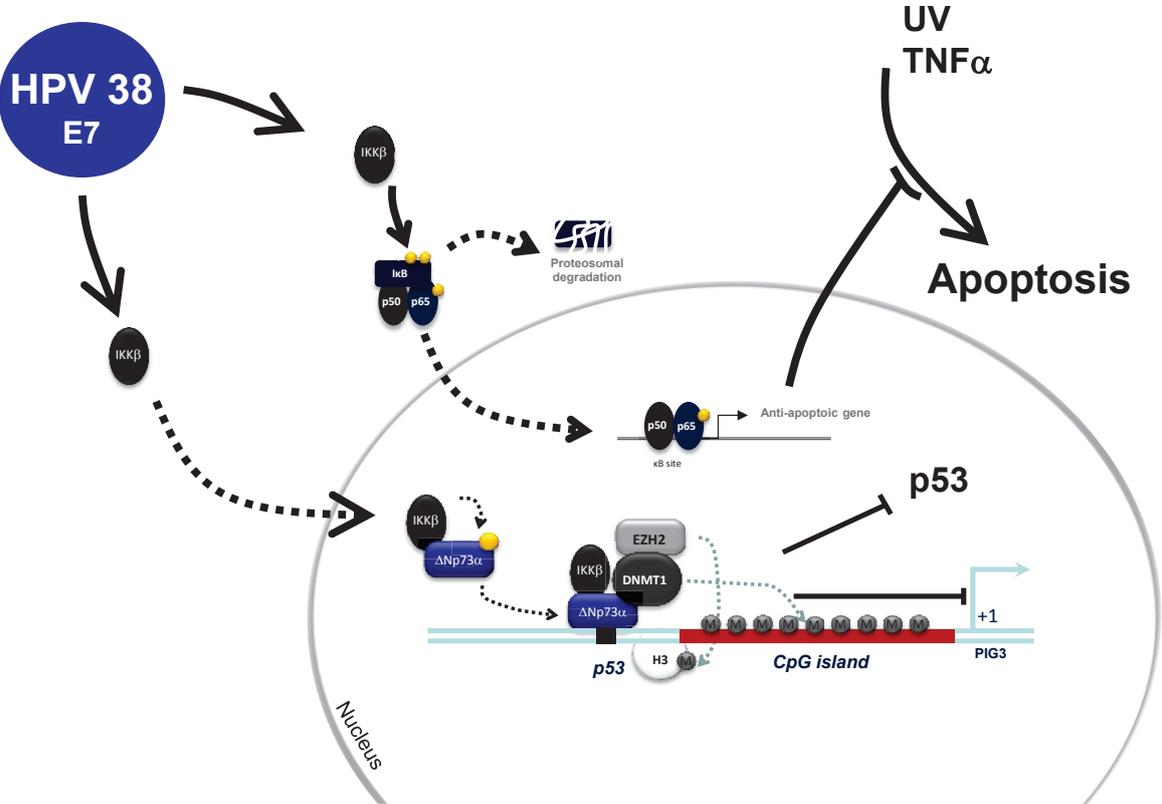


Figure 4. IKK β sub-cellular localization in cancer cells
Immunofluorescence staining for IKK β in head and neck (HNC124, HNC136), breast (Cal51, HBL100, HCC1937) and colon (Co-115, TC7, LoVo) cancer cells. The nuclei were stained with DAPI.

(HNC124, HNC136), breast (Cal51, HCC 1937) and colon (Co-115, LoVo and TC-7) cancer cell lines were then cultured on cover-slides and stained with a monoclonal anti-IKK β antibody. The immunofluorescence analysis shows that, besides its cytoplasmic localization, IKK β is also localized in the nucleus of most of the cancer cells tested (Figure 4). Hence, IKK β nuclear localization appears to be a shared event between some viral- and non-viral- immortalized cells.

Chapter III: Discussion and conclusions

Through sophisticated mechanisms affecting important cellular signaling, oncogenic viruses display an ability to prevent host cell suicide. Among the large number of factors that are involved in the modulation of apoptosis, p53 and NF- κ B signaling are particularly targeted by oncogenic viruses. The present work highlights key roles for HPV38 E7 protein in the prevention of apoptosis. Indeed, expression of E7 protein in human keratinocytes leads to a dual effect toward inhibition of transcription of pro-apoptotic and activation of transcription of anti-apoptotic genes. HPV38 E7 is shown here (i) to activate NF- κ B pathway and subsequent expression of anti-apoptotic genes upon TNF- α treatment or UV-exposure and (ii) to stabilize Δ Np73 α , antagonizing the transcription of anti-proliferative genes regulated by p53.



Model : Schematic representation of summarizing the combined results of our studies.

Although it is evident that HPV38 E6E7 can activate canonical NF- κ B signaling, the molecular mechanisms responsible for IKK kinase activity activation are not yet established. We have observed that E7 protein can bind IKK β but we do not have evidences of the consequences of this interaction. It was shown that other viral protein could also target the IKK complex. For

instance, HTLV1 transforming protein Tax constitutively activates NF- κ B, by physically binding the regulatory subunit of the IKK complex (IKK γ) (248, 249). Therefore, it is possible that the interaction of E7 with IKK β could lead to the stimulation of its kinase activity and consequent stimulation of NF- κ B pathway. The E7 protein domains involved in the interaction with IKK β are still not identified. Their characterization is important for the understanding of the biological significance of this protein complex. Indeed, generation of HPV38 E7 mutants unable to bind IKK β will provide evidence whether the interaction with the cellular kinase is important for the stimulation of its activity.

In our study we have focused on a small number of NF- κ B-regulated genes, but it is likely that a larger number of cellular genes are deregulated. Broader investigation of HPV38-deregulated NF- κ B target genes expression will help to better describe how HPV38 resets NF- κ B to a more virus-advantageous setting, such as transcription of genes involved in the prevention of UV-induced apoptosis.

When NF- κ B signaling is inhibited by different strategies in 38E6E7 HFK, we have observed that inhibition of NF- κ B nuclear translocation had a marginal impact on cellular proliferation, while inhibition of IKK β promoted a severe decrease of cellular growth. This observation highlights the existence of NF- κ B-independent pro-proliferation roles of IKK β . Previous studies have also shown that IKK β activity is not reduced to the unique function of I κ B α phosphorylation and NF- κ B activation. For instance, IKK β has been shown to target several other proteins such as FOXO3a, TSC1, Δ Np73 α , and p53, resulting in the promotion of cell survival and tumorigenesis (250). Further studies will be needed to better dissect the newly discovered IKK β implication in other pathways than NF- κ B. It is also possible that the NF- κ B-independent IKK β -pro-proliferative roles in 38E6E7 HFK could be linked to its subcellular relocalization.

Interestingly, we observed that HPV38 E7 induces translocation of IKK β in the nucleus where (i) it induces stabilization of Δ Np73 α and (ii) it is part of a negative regulatory complex containing Δ Np73 α , EZH2 and DNMT1. In the presented data, we have characterized the

formation of this complex on several p53-regulated gene promoters with the exception of Survivin promoter that is, in contrast to the other investigated genes, an anti-apoptotic p53-target gene. However it is important to explore a larger number of gene promoters that could be bound and regulated by the described protein complex. $\Delta\text{Np73}\alpha$ appeared to be the central component of this transcription inhibitory complex. Investigation of the variation gene expression upon downregulation of $\Delta\text{Np73}\alpha$ expression in 38E6E7 HFK will help to identify the potential target genes and will provide insights on the function of the $\Delta\text{Np73}\alpha$, IKK β , EZH2, and DNMT1 complex.

The interaction of $\Delta\text{Np73}\alpha$ with EZH2 and DNMT1 and its correlation with histone H3Lys27me3 and CpG methylation, reveals a mechanism that can allow an efficient activity of $\Delta\text{Np73}\alpha$ in repressing gene transcription. This event shows that $\Delta\text{Np73}\alpha$ goes over the competitive action against p53, its binding can cause chromatin condensation and stable gene transcription silencing. EZH2 and other polycomb complex 2 components were previously reported to cooperate with DNMTs, including DNMT1, to form transcription inhibitory complexes. In keratinocytes expressing HPV38 E6 and E7, IKK β -phosphorylated $\Delta\text{Np73}\alpha$ appears to hijack this protein complex in order to induce p53-regulated genes silencing. The formation of this multimeric protein complex in 38E6E7 HFK constitutes a mechanism that could also take place in other virus-immortalized cells and non-virus-related cancers. Actually, $\Delta\text{Np73}\alpha$, EZH2, and DNMT1 are found accumulated in several cancers (251-254). In addition, IKK β is also localized in the nucleus of numerous cancer cells lines (Results 3, Figure 4). These observations are in favor of a possible broader cooperation between IKK β , $\Delta\text{Np73}\alpha$, EZH2, DNMT1, and their not yet discovered other partners in the repression of p53-regulated pro-apoptotic genes during cellular transformation. A wider investigation of the gene promoters co-bound by this protein complex will help to better describe their common action within the cells. In addition, it remains of high interest to know if the formation of this complex can be a common event for some human cancers.

As cited above, although IKK β is known to be localized in the cytoplasm, it is also accumulated in the nucleus of 38E6E7 HFK. This subcellular delocalization appears to be induced by the

protein E7. However, the mechanism of IKK β nuclear translocation is not yet known. As previously mentioned, the identification of E7 mutants that lose their ability to bind IKK β will provide evidences on the possible implication of the E7/IKK β interaction in the translocation of IKK β in the nucleus.

A previous report has shown that IKK β , in the nucleus of B-cell non-Hodgkin lymphoma, is part of a transcription regulation complex. In cooperation with BAFF-R, IKK β phosphorylates histone H3 and leads to chromatin remodeling and gene transcription regulation (255). Thus, it is likely that IKK β , in addition to Δ Np73 α , binds to a wider range of transcriptional regulatory factors in 38E6E7 HFK. One possible strategy to evaluate this possibility is to perform ChIP-seq experiments using IKK β antibodies. This experiment will lead to the identification of different promoters that are associated with IKK β . The analysis cis responsive elements within these promoters will provide evidence whether IKK β binds additional cellular transcription factors. Finally, ChIP and Re-ChIP experiments will be needed to validate the ChIP-seq results. It is likely that such an approach will also result in the identification of novel and important genes that are regulated by IKK β in transformed cells.

In summary, the presented data not only further clarify the HPV38 mechanisms in deregulating several cellular events, but also pave the way to novel studies aiming to elucidate the precise events that play a key role in cellular transformation.

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Alteration of p53 and NF- κ B pathways by E7 protein from cutaneous Human Papillomavirus type 38

Abstract

Viral infections contribute to 15–20% of all human cancers. Studying the mechanisms employed by the oncogenic viruses to induce cellular transformation is essential for a better understanding of the resulting cancers and the discovery of new mechanisms involved in cancer development which can be targeted in therapeutic approaches. Human papillomaviruses (HPVs) are small dsDNA viruses which have been clearly associated with certain cancers. They were first isolated from the skin of patients suffering from Epidermodysplasia Verruciformis (EV) having an increased susceptibility to infection by specific HPV types and to the development of non-melanoma skin cancer (NMSC). Certain cutaneous HPV types, such as 5, 8, and 38, are suspected to play a role in skin cancer development. However the direct role of cutaneous HPV in the etiology of cancer is still under debate. Previous studies from our laboratory have reported that HPV38 E6 and E7 proteins are able to immortalize human primary keratinocytes *in vitro* and *in vivo*. Cellular immortalization can be achieved through the deregulation of important signaling pathways including p53 and NF- κ B. In the present work, we have investigated the molecular mechanisms of p53 and NF- κ B pathways deregulation by E6 and E7 oncoproteins from HPV38 in human keratinocytes. We show here that HPV38 E6E7 induce the formation of a transcription repressor complex including IKK β , Δ Np73 α , and polycomb group members EZH2 and DNMT1. The formation of this protein complex correlates with the inhibition of several p53-target genes, such as PIG3. We also report in these studies that HPV38 E6E7 activate NF- κ B pathway, which plays an important role in the survival of HPV38 E6E7-immortalized human keratinocytes upon TNF- α – and UVB-mediated apoptosis. In addition our data highlight E7 being the main HPV38 protein mediating p53 and NF- κ B deregulation. Our studies shed light on novel molecular mechanisms that could be important for HPV38-mediated cellular transformation.

Dérégulation des voies de signalisation p53 et NF- κ B par la protéine E7 du Papillomavirus Humain de type 38

Résumé

Les infections virales sont responsables de 15 à 20 % des cancers humains. L'étude des mécanismes moléculaires avec lesquels les virus oncogènes induisent la transformation cellulaire est essentielle pour la compréhension des cancers qui en résultent. Cela permettra également la découverte de nouveaux mécanismes pouvant être impliqués dans le développement de cancers, qui peuvent être ciblés par des approches thérapeutiques. Les virus du papillome humain (HPV) sont des petit virus à ADN qui fut isolés de la peau de patients souffrants de Epidermodysplasia Verruciformis (EV) qui cause un risque élevé d'infection par les HPV et le développement de cancer de la peau non mélanique (NMSC). Certains HPV cutanés, tels que HPV5, 8 et 38, sont suspectés de jouer un rôle dans de développement du cancer de la peau. Cependant, le lien direct entre les HPV cutanés et l'étiologie du cancer n'est pas encore clairement établi. Des études de notre laboratoire ont montré que les oncoprotéines HPV38 E6 et E7 sont capables d'immortaliser des kératinocytes primaires humains *in vitro* et *in vivo*. Pour immortaliser des cellules, d'importantes voies de signalisations, telles que les voies de p53 et celle de NF- κ B, doivent être affectées. Dans cette étude, nous avons cherché à mettre en évidence les mécanismes moléculaires menant à la dérégulation de p53 et de NF- κ B par E6 et E7 de HPV38, dans des kératinocytes humains. Nous avons montré que HPV38 E6 et E7 induisent la formation d'un complexe protéique incluant IKK β , Δ Np73 α , EZH2 et DNMT1. La formation de ce groupement protéique corrèle avec l'inhibition de la transcription de certains gènes cibles de p53, tel que PIG3. Nous avons également mis en évidence l'activation de la voie NF- κ B par les oncoprotéins E6 et E7 de HPV38. Cette activation est importante par le rôle joué par NF- κ B dans la protection des cellules de l'apoptose induite par TNF- α et par l'exposition aux rayonnements UVB. De plus nous avons observé que E7 est la principale oncoprotéine de HPV38 responsable de la dérégulation des voies p53 et NF- κ B. Nos études mettent en évidence de nouveaux mécanismes moléculaires qui peuvent être essentiels dans le processus de transformation cellulaire par HPV38.