Reprogramming of skin keratinocytes by the E7 oncoprotein of HPV8

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..... In memory of my father who lost his battle against cancer on the 8th of December 2020 at the age of 70.

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I. Summary

The human papillomavirus type 8 (HPV8), an oncogenic member of the genus betapapillomavirus (betaHPV), is associated with the development of actinic keratoses and squamous cell carcinoma of the skin. The viral early proteins E6 and E7 are oncoproteins which are known to interact with important regulatory mechanisms of infected keratinocytes. However, the molecular details how this occurs in case of HPV8-E7 is still only poorly understood. Previous research of our group had already shown that HPV8-E7 plays a pivotal role in inducing and regulating keratinocyte proliferation and invasion. It was also shown that the extracellular matrix protein fibronectin and the integrin α3β1, expressed on HPV8 positive keratinocytes, is key in regulating the invasive behavior of these cells. Furthermore, we had shown that fibronectin is not only overexpressed in HPV8-E7 positive keratinocytes, but also found to be enriched in the peri-tumoral stroma of betaHPV-positive skin cancer. Therefore, the overall aim of this thesis was to arrive at a deeper understanding how HPV8-E7 reprograms the cell and how it hijacks vital cellular processes and how this interference may ultimately contribute to malignant transformation processes in HPV8 infected keratinocytes. To extend our knowledge of how these events occur transcriptomic, total and phospho-proteomics as well as protein-protein interaction assays were employed.

cDNA microarray results led to the observation that global gene expression changes induced by HPV8-E7 are mainly accomplished through binding sites within the gene promoters for the transcription factors Sp1/Sp3. In transient transfection assays we could confirm that HPV8-E7 stimulates the activity of promoters of cellular genes which contain Sp1/Sp3 binding sites, including the fibronectin gene promoter. Interestingly the HPV8-E7^{L23A} mutant, which is not able to trigger keratinocyte invasion was unable to activate the fibronectin promoter **(Kirschberg*, Heuser* et al., 2019)**.

By using the same cDNA microarray results GADD34 (growth arrest and DNA damage-inducible protein 34) and GDF15 (growth/differentiation factor 15) were identified as novel downstream genes targeted by E7. In addition, proteomic analyses of HPV8-E7 positive keratinocytes cultivated on a fibronectin matrix showed that the presence of E7 led to hyper- or hypo-phosphorylation of cellular proteins. Here, we also revealed that hypo-phosphorylated proteins are mainly associated with DNA damage repair and DNA replication, whereas hyper-phosphorylated proteins are

apparently important for cytoskeletal organization and also include proteins associated with cell polarity. Therefore, these observations reveal that signals downstream of fibronectin resulting in hyper-phosphorylation of proteins are critical for cell invasion. Interestingly, among the identified up-stream kinases were the proto-oncogenic Src-kinase family members Lyn and Fyn, which are over-activated in the presence of HPV8-E7 **(Kirschberg et al., 2021)**.

Looking at direct E7 interacting proteins, NuMa (nuclear mitotic apparatus protein 1) and ATP5B (ATP synthase F1 subunit beta) were identified as novel HPV8-E7 interacting partners. E7 appears to co-localize with NuMa in mitotic cells. Curiously, in a small subset of these cells E7 was only found at one whereas NuMa was found at both spindle poles. This could implicate that the binding of E7 to NuMa at only one spindle pole might also affect the ratio of symmetric / asymmetric cell division of epidermal progenitor cells, which could explain the already known enrichment of stem-cell-like cancer cells in HPV8 positive primary keratinocyte cultures **(Oswald*, Kirschberg* et al., 2019)**. The observed interaction of E7 and ATP5B was most intriguing as betaHPV E7 had been mainly known as a nuclear and cytoplasmic protein. The ability to bind ATP5B is not exclusive for HPV8 but could also be shown for low-risk (HPV11) and high-risk (HPV16) mucosa -infecting HPV. The association of E7 with ATP5B was accompanied by an increase in mitochondrial energy production. In particular E7 positive keratinocytes showed a stark increase in their spare respiratory capacity, allowing the cells to meet their heightened energy demands. The influence of ATP5B manipulation was further analyzed in oropharyngeal cancer patient groups which included both HPV16 positive and HPV-negative tumors. Interestingly, HPV-positive cancers showed a far more favorable outcome compared to HPV-negative tumors if ATP5B expression was high **(Kirschberg et al., 2020)**. Having identified these new interaction partners, namely NuMa and ATP5B, it appears that E7 exerts its oncogenic functions in various cellular compartments.

Virtually all so far published studies on the oncogenic functions of E7 were conducted in cells expressing the viral oncoproteins individually. Interestingly, when HPV8-E7 is co-expressed with HPV8-E6 other oncogenic functions appear to become active, leading to the CHK1 (checkpoint kinase-1) protein degradation. This disruption of CHK1 was mediated by manipulating the autophagic pathway that was so far not known to be a degradation pathway used by HPV oncoproteins **(Akgül, Kirschberg et al., 2019)**.

In summary, the results of this thesis provide novel insights from different experimental angles, which could very well be relevant for the rise of tumorigenic cells in betaHPV infected skin. Further studies reversing these E7-mediated effects might be invaluable for future treatment strategies for HPV-mediated cancers.

II. Introduction

2.1 Oncogenic viruses in humans

Carcinogenesis is a multi-step process that encompasses the accumulation of multiple DNA damage events that are often referred to as oncogenic “hits”. Most commonly these events take place either spontaneously or as a result of environmental factors (e.g. UV radiation) that lead to alterations of the genome. Having said that these key elements drive tumor development, allowing for further DNA damage which ultimately result in the disruption of essential cellular pathways leading to deregulation of normal cell function and paving the way for cancerous growth (Banks et al., 2012). Classic hallmarks of cancer have been identified over the past decades and include aberrant induction of new blood vessel formation, hyperproliferation, invasion of surrounding tissue and cellular immortality as well as their ability to evade the immune system, which also includes their inability to respond to cell death commands issued by the host immune system (Hanahan and Weinberg, 2000;Schiller and Lowy, 2014).

Oncogenic virus	Abbreviation	Associated cancer(s)
Human papillomavirus of genus alpha	**HPV***	Cervical cancer, anal cancer, vaginal cancer, oropharyngeal cancer
Human papillomavirus of genus beta	**HPV**	Skin cancer in Epidermodysplasia verruciformis patients
Merkel-cell polyomavirus	**MCPyV**	Merkel cell carcinoma
Human T cell lymphotrophic virus, type-1	**HTLV-1***	Adult T-cell lymphoma
Epstein-Barr virus	**EBV***	Burkitt lymphoma, Hodgkin lymphoma, non-Hodgkin lymphoma, nasopharyngeal cancer, T-cell and NK lymphoma
Kaposi’s sarcoma herpes virus	**KSHV***	Kaposi’s sarcoma, primary effusion lymphoma
Hepatitis B virus	**HBV***	Hepatocellular carcinoma
Hepatitis C virus	**HCV***	Hepatocellular carcinoma

Table 1. List of known human tumor viruses, associated cancers and involved viral oncogenes. The virus type is described with its abbreviation, its associated cancer and the corresponding virus oncogene. In addition, the asterisks indicate the members of the Group 1 carcinogens defined by the International Agency for Research on Cancer (Ajiro and Zheng, 2014;Howley and Pfister, 2015).

The presumption that viruses may be cancer-causing agents emerged in the early 20th century with the independent discovery of cell-free transmission of human warts by Ciuffo (1907) (reviewed in (zur Hausen, 2009)), by Ellerman and Bang (1908) who performed research on chicken leukaemia and Rous (1911), who conducted experiments related to chicken sarcoma (reviewed in (Moore and Chang, 2010)). Following the identification of v-src as a viral oncogene for Rous sarcoma the hypothesis that viruses may cause cancer was finally confirmed in 1976 (Stehelin et al., 1976). The first actual direct proof of virus involvement in human carcinogenesis emerged in 1964 when a direct link between a host virus infection and carcinogenesis was discovered with the identification of the Epstein-Barr virus as the causative agent for Burkitt lymphoma (Epstein et al., 1964) and later the identification of several other oncogenic viruses including the Merkel cell polyomavirus and high-risk human papillomaviruses (HPV) as the causative agents for cervical cancer in women (Mesri et al., 2014). A typical property of most virus-associated cancers is that cells undergo a malignant transformation as a result of cancer-promoting virus genes, which then interfere with cellular processes that are also important for completion of the virus life-cycle. Usually infections with such viruses also persist and are not cleared by the host, although this is not true for all carcinogenic viruses (Ajiro and Zheng, 2014). In 2012 the International Agency for Research on Cancer (IARC) characterized eight viruses as carcinogenic based on various epidemiological, clinical and a number of studies (table 1). We now know that approximately 12-15 percent of all cancers are caused by these oncogenic viruses, which are listed in the table above together with their respective oncogenes (Bouvard et al., 2009;Mavropoulos et al., 2014;Mesri et al., 2014;Harwood et al., 2016).

2.2 Papillomaviruses (PV)

2.2.1 Phylogenetic evolutionary relationship between the different PV types

Papillomaviruses (PV) are known to infect a variety of different vertebrate species. Up to now more than 400 PV have been discovered in a broad range of vertebrates and subsequently fully sequenced. PV are a vast family of viruses that is comprised of five genera designated alpha, beta, gamma, mu and nu (de Villiers et al., 2004). The genus gamma includes the majority of the known HPVs, witch researchers constantly finding previously unknown PV (Gheit, 2019). Out of the known PV more than 200 have humans as their primary host (HPV) (Fig. 1).

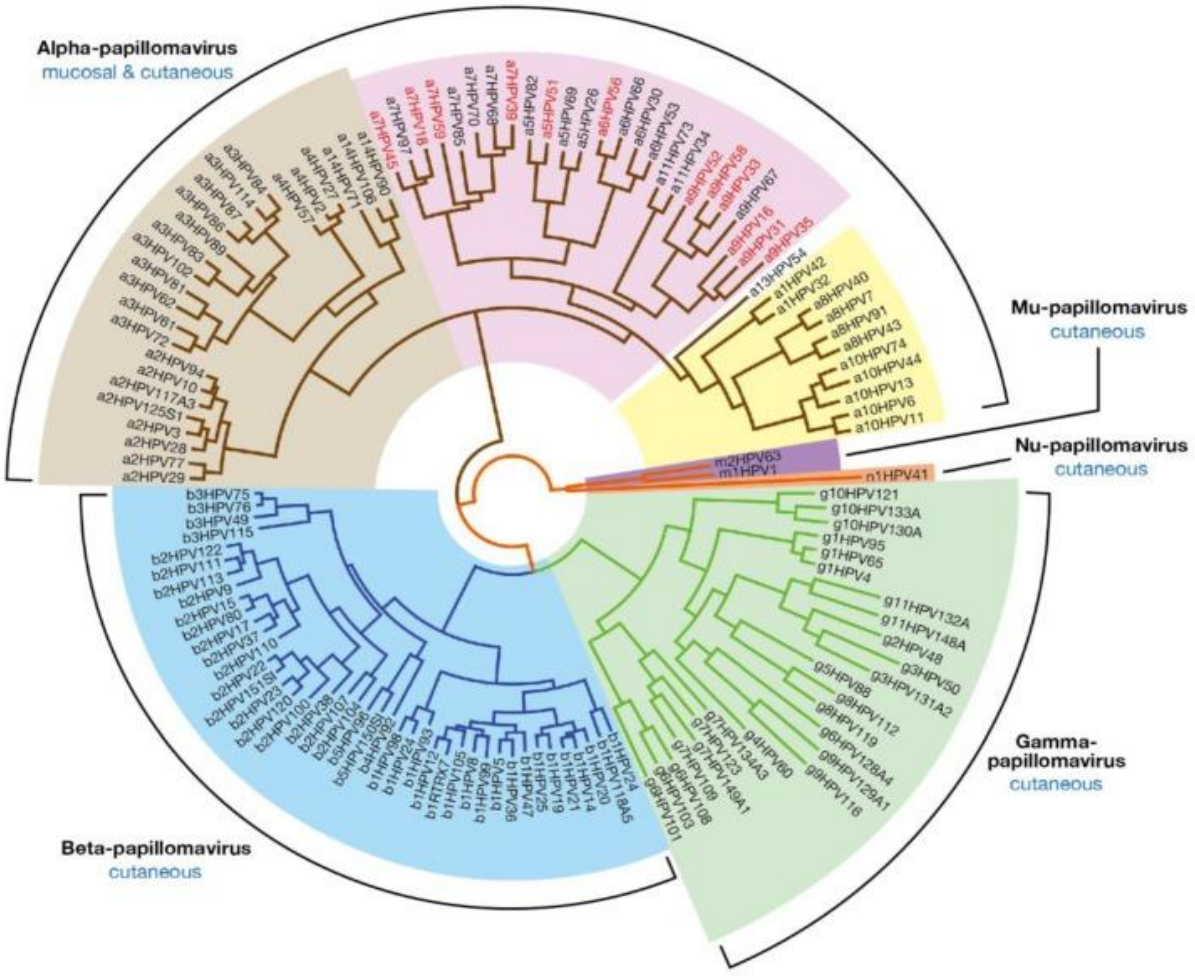

Fig. 1: Phylogenetic tree of the papillomavirus family. The papillomavirus family is sub-divided into 5 genera. Some alpha papillomaviruses are known to be “high-risk”, and some beta papillomaviruses are associated with skin cancer. The high-risk types highlighted in red are confirmed as “human carcinogens” on the basis of epidemiological data. Although the primary tissue associations of each genus are listed as either cutaneous or mucosal, these designations do not necessarily hold true for every member of each genus. The evolutionary tree is based on alignment of the E1, E2, L1, and L2 genes (Doorbar et al., 2012). The data this overview is based on was taken from the PaVE library that contains all known papillomaviruses across different species (Van Doorslaer et al., 2017). The fact that more than 200 different HPV are known to infect humans is highly indicative of a long virus-host relationship (Egawa et al., 2015;Van Doorslaer et al., 2017).

At present (March 2021) 218 human papillomaviruses (HPV) types with reference numbers have been characterized and additionally more than 200 types are known but have not yet received an HPV type number (Fig.1) (Egawa et al., 2015;Van Doorslaer et al., 2017). It is accepted that one key characteristic of PVs is their host specificity and that they only infect clearly defined anatomical compartments (such as the mucosal or cutaneous skin) where these viruses may give rise to benign or also malignant lesions (Doorbar et al., 2015). The pathological profiles range from hyper-proliferative warts up to precursor lesions, the latter of which may progress to malignant cancers. For instance, the association of “high-risk” alphaHPV types with cervical cancer is by now well established and has led to vaccination efforts against HPV16 and 18, which are primarily responsible for cervical cancer. In addition they are also known to be the causative agents for anal, penile, and orophangyeal cancers. AlphaHPVs are also unique insofar as they are the only genus of the family which does not only infect cutaneous but also mucosal tissues. Most HPV however are efficiently controlled by the immune system, even when they are detected in skin swabs of immunocompetetent individuals (Poljak et al., 2009;Bottalico et al., 2011;de Koning et al., 2015). However, while the phylogenetic species concept as depicted in Fig. 1, can be useful it is very difficult to implement as for many papillomavirus types very little information is available regarding their biology and they are only vaguely characterized (Egawa et al., 2015).

2.2.2 Viral structure

HPVs are small (Ø 55nm), icosahedral non-enveloped DNA viruses. The encapsulated virion is comprised of 72 capsomers and contains the circularized double-stranded viral DNA which contains about 8,000 base pairs (bp). The circular DNA is associated with the cellular histones H2a, H2b, H3 and H4 in infected host cells, forming a structure of about 8 nm very similar to nucleosomes (Favre et al., 1997) (Porter et al., 2021). The genome is further functionally organized into a coding region which typically encompasses eight overlapping open reading frames (ORFs) and a non-coding region (NCR) (Graham, 2010). Genes within the coding region are numbered according to their expression order during the viral life cycle and are further divided into early (E) and late (L) genes.

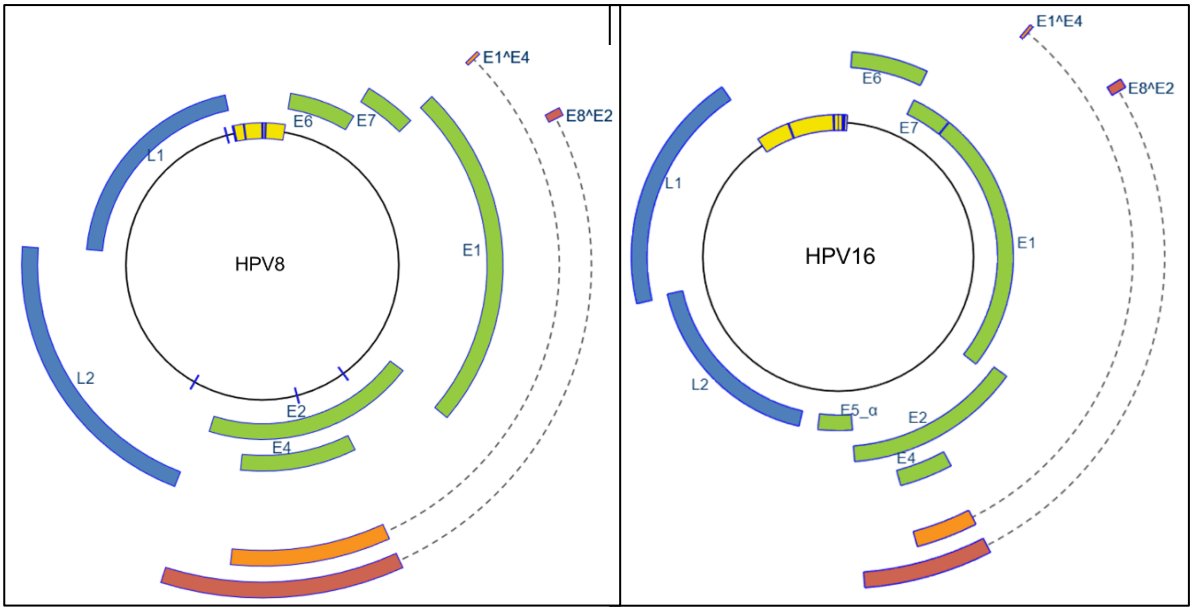

Fig. 2 Circular genome structure of HPV8 and HPV16. The HPV16 DNA (right hand image) contains the genes for E1, E2, E4, E5, E6, E7 (green), late genes L1, L2 (blue), a non-coding region (NCR, yellow) and encodes for two splice variants E1^E4 / E8^E2 (orange / red) and has a total size of 7906 bp. The HPV8 DNA (left hand image has a size of 7654 bp and contains most ORFS corresponding to these genes but lacks the early gene E5. Source: (Van Doorslaer et al., 2017) http://pave.niaid.nih.gov).

While the early genes encode for proteins governing transcription, DNA replication and host cell transformation processes, the late regions carry the genes for the viral capsid production required for virion assembly and subsequent release (Fig. 2).

Whether a viral infection remains latent or becomes active is dependent on gene expression or repression. This process is tightly controlled by the viral origin of replication (ori), an enhancer element specific for keratinocytes, which works in concert with an early and late promoter in the NCR between the ORFs of the L1 and E6 genes. Once transcription has been initiated the host cells begin transcribing the polycistronic viral mRNAs (Olmedo-Nieva et al., 2018). In case of HPV 16, early genes are under the control of the early promoter which governs transcription of E1, E2 as well as E6 and E7, all of which are required for maintenance of the viral genome in the dividing basal cells. The early proteins E1 and E2 (a helicase) are expressed, with E1 guiding the transfer of E2 to the host replication machinery where they bind to the *ori* of replication (Genders et al., 2015;Spriggs and Laimins, 2017). During the vegetative phase of the infection the expression of regulatory viral proteins including E4 and E5 (only some genera) increases. Typically, during ongoing virus gene expression the cell cycle exit becomes blocked. Thus, the of S-phase is induced again by the early proteins E6 and E7 (Moody and Laimins, 2010), which results in the re-entry of infected keratinocytes into the cell cycle, allowing for their genome amplification (Genders et al., 2015).

It needs to be said thou that gene expression in betaHPV may become deregulated, i.e. in association with UV irradiation, particularly in immunosuppressed patients (Genders et al., 2015;Howley and Pfister, 2015). In high-risk alphaHPV, E6 and E7 allow for extensive cell proliferation associated with neoplasia, whereas in betaHPV cell cycle re-entry and genome amplification is maintained without neoplasia. The late promoter only becomes active upon cell differentiation and exerts control over the late viral genes, which results in the expression of E1, E1ˆE4, E2, E5 (only some genera), and the L1 and L2 capsid proteins. While sequencing efforts of different HPVs have revealed a great diversity in the encoded genes the process of gene transcription itself is strongly conserved (reviewed in (Spriggs and Laimins, 2017)). Furthermore, it was shown by Doorbar et al. in 1991 already that alternative splicing is involved, which creates the fusion protein E1^E4. This protein is required for dissolving the cytokeratin matrix of the host cell allowing for virus release (Doorbar et al., 1991). The E8^E2C protein represents a splice variant of the E8 and E2 ORF and serves as a replication repressor (Sankovski et al., 2014).

2.2.3 HPV life cycle

In general HPV keratinocytes located in the basal layer within stratified squamous epithelia of either mucosal or cutaneous origin are infected through micro-lesions of the skin. These basal cells are the least differentiated cells and the only cells which are actively proliferating within the epithelium. Since the virus life cycle is strictly coupled with the differentiation status of cells and requires proliferating cells to propagate the infection, these cells are specifically targeted by binding to cell surface receptors and elements of the extracellular matrix (ECM) to facilitate virus uptake into host cells (Moody and Laimins, 2010). During initial infection it has been shown that HPV predominantly binds to ECM components which contain many proteoglycans, particularly heparan sulfate proteoglycans (HPSG). These proteins are glycoproteins containing one or more covalently linked heparan sulfate chains. HPV capsids directly interact with these structures via the L1 protein, leading to conformational alterations in both L1 and L2 proteins (Richards et al., 2013). Additionally, several studies have also observed an interaction with laminin-332, which is also associated with wound healing and thus used as a target of HPV (Richards et al., 2014;Kines et al., 2016). Following the conformational changes, the virus particle binds to different uptake receptors (Day et al., 2008).

Following these specific conformational changes, which likely reduce the affinity to HPSG, the virion associates with subsequent non-HSPG uptake receptors including syndecan-1 (Day et al., 2008;Surviladze et al., 2012). Numerous non-HSPG candidate receptors for HPV entry have meanwhile been identified including the cytoskeletal adaptor obscurin-like 1 (OBSL1), tetraspanins, growth factor receptors, and annexin A2 (Kines et al., 2016;Wustenhagen et al., 2016;DiGiuseppe et al., 2017). The transmembrane integrin α6β4 is involved in virus uptake as well, serving as an additional co-receptor used by the virus for homing in on target cells for internalization (reviewed in (Mikulicic and Florin, 2019).

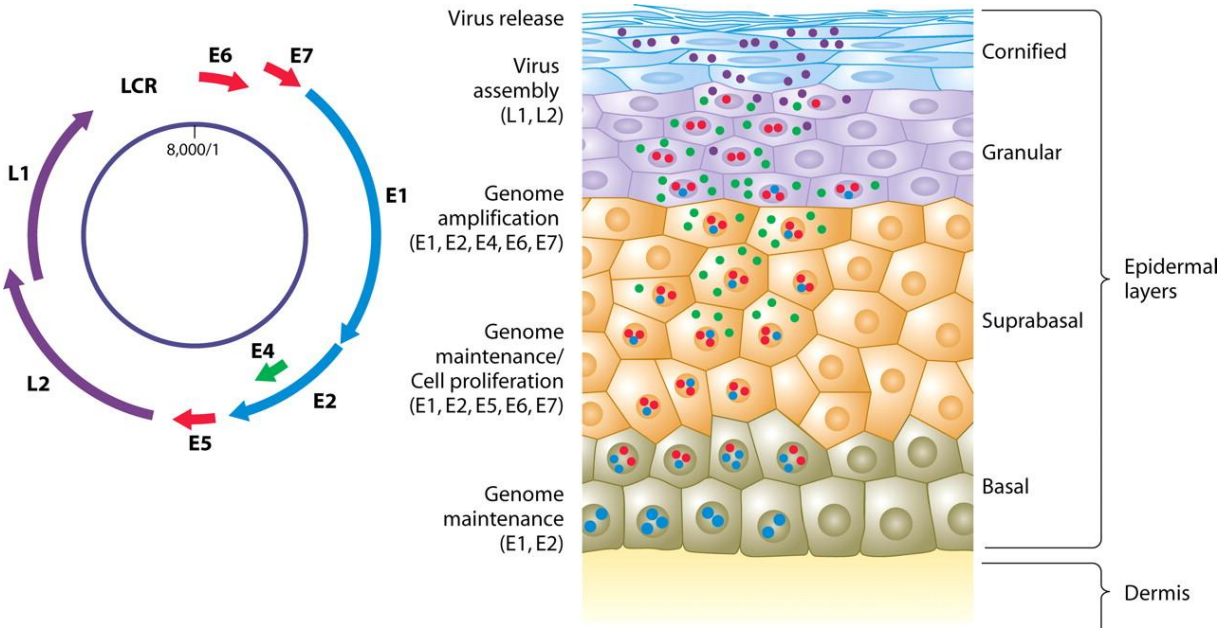

Fig. 3 Life cycle of HPV. The left-hand image shows another color-coded representation of the circular HPV DNA which matches the colored dots in the right hand image. HPV infects basal cells after gaining entry via micro lesions. In the lower epithelial layers E1, E2, E4, (E5), E6 and E7 are expressed. Expression of virus proteins progressively increases in the upper epidermal layers as completion of the virus life cycle is tightly coupled with host cell differentiation and proliferation, and both processes are de-regulated to maintain proliferation. This is accompanied by vegetative viral DNA replication. The expression of the late genes occurs in terminally differentiated cells in the granular layer, where also the virus assembly takes place. The virions are then released through skin shedding from the cornified layer. Should expression of the oncogenes E6 and E7 become abnormally high, the suprabasal layer will become enlarged as a result of disruption of physiological host cell differentiation (Lazarczyk et al., 2009).

Over the millennia both alphaHPV and betaHPV (see Fig. 2 for their genomes) have developed differing strategies to complete their respective life cycles within their human hosts (Bravo and Felez-Sanchez, 2015). While both viruses gain entry to undifferentiated basal cells via micro lesions, alphaHPV preferentially target mucosal epithelia such as the cervix, or anogenital region and the oropharynx where the virus may also cause a persistent infection by integration of the virus DNA into the host genome. On the long run this event can subsequently lead to progression to cancerous lesions in the case of infections with “high-risk” oncogenic alphaHPV, the process of

which will be described in further detail below. In contrast, while betaHPV also infect basal epithelial cells they are mainly found in cutaneous tissues. Most recently, betaHPV have also been found in the anal canal and oral mucosa in men who have sex with men, as well as HIV patients (Gheit et al., 2020;Galati et al., 2021). The infection with betaHPV usually remains asymptomatic in the general population. Furthermore, betaHPV do not have the ability to integrate their DNA into the host genome and their DNA is exclusively found as episomes in infected cells (You, 2010;Quint et al., 2015;Doorbar, 2016). Once the virus has entered the cell the expression of E1 and E2 allow for induction of viral DNA replication (Akgül et al., 2003). This is achieved by binding to the viral origin of replication and hijacking the host cell DNA replication complex by these two early proteins. In this context E2 may exhibit either activating or repressing functions regarding transcription (Van Doorslaer et al., 2013), and the protein also governs the distribution of virus DNA during mitotic processes by attaching viral episomes to host cell chromosomes in infected cells (Sekhar and McBride, 2012;Della Fera et al., 2021).

Over the course of an HPV infection the physiological cell cycle can become disrupted by aberrant expression of the early proteins E6 and E7 (Hufbauer and Akgül, 2017). The expression of these two oncogenes is required to substitute for the absent host cell replication machinery in suprabasal cell layers (Fig. 3) and also enhances cell proliferation, delay of cell cycle progression and counteracts apoptotic processes (Ghittoni et al., 2010;Spriggs and Laimins, 2017). In the case of “high-risk” alphaHPV the combined over-expression of E6 and E7 (often, but not always as a result of integration of the virus genome into the host cell, which results in aberrant E6 and E7 expression) may unhinge various cellular checkpoints as they interfere with vital regulatory processes involved in cell cycle progression to ensure that cells retain their S-phase competence while differentiating, an ability that would normally be abolished during host cell differentiation. In host cells with a persisting infection this inevitably leads to an accumulation of more and more DNA damage and as such infected tissues may progress further to transformations including malignant neoplasia (Albert and Laimins, 2020). The E4 protein is expressed before the capsid proteins L1 and L2 and plays a pivotal role in cell cycle progression and exit. It is therefore essential for virus release and further virus transmission (Doorbar, 2013). Cutaneous HPV such as the betaHPV type 8 can be detected in newborns as early as a few weeks following their birth, implicating that initial infection with HPV must happen early in life (Antonsson et

al., 2003;Hasche et al., 2018). The expression of betaHPV oncogenes E6 and E7 results in cell cycle re-entry and genome amplification without neoplasia in immunocompetent individuals. However, when their expression is associated with ultraviolet (UV) irradiation and/or immunosuppression, non-melanoma skin cancers can arise (Akgül et al., 2003;Howley and Pfister, 2015;Quint et al., 2015;Hufbauer and Akgül, 2017). During the vegetative phase of a betaHPV infection expression of E4 is upregulated in the stratum spinosum. In higher suprabasal layers upregulation of virus gene expression is increased and the capsid proteins L1 and L2 (Fig. 3) are expressed in the cornified layer to allow for virus assembly and release by shedding of keratinocytes. The physiological reservoir for latent infections with betaHPV seems to be hair follicular stem cells. A thick lymphocyte infiltrate generally obscures the connection between lymphoid and epithelial compartments and as such compartmentalizes the epithelial sheath into irregular nests and cords. This intertwined epithelial structure may therefore allow for virion access to tissue stem cells at the immune-privileged stem cell niche, which is characterized by an inhibition of virus-specific T cell activity, and thus may aid the virus in immune evasion during the initial HPV infection as well as subsequent virus-mediated malignant transformations (Borgogna et al., 2012;Egawa et al., 2015;Quint et al., 2015;Olivero et al., 2018;Tampa et al., 2020). BetaHPV infections are mostly asymptomatic in immunocompetent individuals, with betaHPV primarily infecting slow-cycling stem- or stem-like cells of the epidermis and slowly spreading throughout the basal layer as cells divide with the viral DNA persisting exclusively in cells carrying the virus episomes (Quint et al., 2015).

2.2.4 High-risk alphaHPV and their role in tumor development

AlphaHPV are subdivided into low-risk and high-risk and are sexually transmitted. Having said that up to 80% of the human population become infected at some point during their life, yet the infection is usually efficiently cleared by the host immune system within a few months post exposure (Bouvard et al., 2009). The real problem arises from persistent infections which can give rise to cancer precursor lesions, which ultimately result in carcinomas. These infections are often accompanied by genomic integration of the virus DNA into the host genome, which results in continuous aberrant E6 and E7 over-expression while the expression of all other virus genes is abolished. As a consequence, both E6 and E7 interfere with a number of pathways, including DNA damage repair and cell cycle progression pathways as well as cell proliferation

(Ajiro and Zheng, 2014;Hübbers and Akgül, 2015;Spriggs and Laimins, 2017). Here, cervical cancer is the most well-known HPV induced cancer and second most common cause for cervical cancer in women (Wieland et al., 2014), with more than 95% of tumors of the cervix and 20-30% of oropharyngeal squamous cell carcinoma (OPSCC) being caused by a persistent infection with HPV16 and HPV18. It was first proposed in 1983 that HPV may be the driving factor for cancers of the head and neck region, a hypothesis formed on the observation that these tumors showed a clinical profile similar to that of anogenital cancers caused by HPV and the already back then known preference of HPV for infection of epithelial cells of either cutaneous or mucosal origin (Syrjanen and Syrjanen, 2008). Every year there are about 270,000 head and neck cancer (HNSCC) cases worldwide. In 50% of these cases high-risk alphaHPV are the causative agents for these cancers, particularly HPV16 (90%; (Kreimer et al., 2005). Most of such HPV16-mediated tumors are OPSCCs found at the tonsils or the base of the tongue. An increased incidence of OPSCC has been described in many developed countries and found to be in correlation with a profound rise of HPV-positive cases (Würdemann et al., 2017). The major risk factors for HPV-negative OPSCC are alcohol consumption and tobacco smoking. HPV-mediated OPSCC are characterized by a distinct gene expression profile that significantly differs from OPSCC related to tobacco or alcohol abuse (Martinez et al., 2007). Retrospective analyses of many clinical trials have demonstrated that patients with HPV-mediated OPSCC generally have a better prognosis and long-term survival following removal of the tumor tissue. Furthermore, they also respond more favorably to standard radio-chemotherapy treatment than patients with HPV-negative tumors (Klussmann et al., 2009;Nasman et al., 2017;Chera and Amdur, 2018). How HPV may be involved in other HNSCC cases is still being investigated (Hübbers and Akgül, 2015).

2.2.5 BetaHPV and their role in non-melanoma skin cancer

With up to three million cases per year non-melanoma skin cancer (NMSC) is the most prominent skin cancer variant in Caucasians with rising incidence rates world-wide over the course of the last 40 years (Zink, 2019). Australians are most affected which is likely due to increased UV irradiation as a consequence of the thinning ozone layer over the Australian continent (Lomas et al., 2012). These cancers are typically derived from keratinocytes (Small et al., 2016) and can be further sub-divided into Merkel-cell carcinomas, basal and squamous cell carcinomas (BCCs or SCCs, respectively)

(Eisemann et al., 2014). These tumors have a broad range of pathological features ranging from slowly progressing to being highly invasive and as such also different clinical outcomes (Hasche et al., 2018). Up to 97% of invasive SCCs are associated with malignant progression of actinic keratoses (AK) which represent precursor lesions of SCCs. However, there is a likelihood that these lesions may progress and become SCCs with cancer cells invading basement layers of affected tissues, allowing the tumors to advance further into the dermis and metastasize (Yanofsky et al., 2011).

It was in patients suffering from the rare hereditary genetic defect Epidermodysplasia verruciformis (EV) that the carcinogenic potential of an infection with betaHPV (in particular HPV5 and HPV8) was shown as betaHPV are present in the skin in high viral DNA loads with high oncogene expression in skin SCC (Orth, 2006). Studies have reported that precursor lesions such as AKs advance in 30-60% of the cases to SCC in EV patients (Howley and Pfister, 2015). The genetic cause of EV lies in mutations of at least three genes, namely the homologous transmembrane channel-like (TMC) proteins TMC6 (EVER1) and TMC8 (EVER2). Both proteins are important for zinc homeostasis through interaction with the Zn_2C transporter protein ZnT-1 and also found in cells other than keratinocytes (Lazarczyk et al., 2009;Lazarczyk et al., 2012). The third gene, encoding for the calcium and integrin-binding protein 1 (CIB1) was recently identified as another genetic mutation. CIB1 forms a complex with EVER1 and EVER2, while CIB1 is not expressed in EVER1- or EVER2-deficient cells. EVER1 and EVER2 are not impaired in their function in human keratinocytes lacking CIB1. In keratinocytes the CIB1 protein interacts with the HPV E5 and E8 proteins encoded by alphaHPV and gammaHPV types, suggesting that this protein acts as a restriction factor against HPV (de Jong et al., 2018a). BetaHPV were also detected in SCC of non-EV patients, although viral loads in these patients are usually much lower than in EV patients. In organ transplant recipients (OTR) the frequency of cutaneous SCC development is increased by up to 65 - 250 times compared to the general populace, with the onset of tumorigenesis (measured in years after organ transplantation) being paralleled by high viral loads (Fig. 4) (Nindl and Rösl, 2008). It is also important to note that a detectable sero-response for one or more betaHPV types around the time of receiving an organ transplant serves as a predictive marker for increased risk to develop SCCs (Genders et al., 2015). OTRs frequently exhibit field cancerization of the skin, which are then the point of origin for multiple HPV-driven SCCs (Nindl and Rösl, 2008;Vinzon and Rosl, 2015).

In immunosuppressed patients the HPV DNA prevalence in cutaneous SCC was found to be much higher. These patients can develop the clinical features of EV, which is then called “atypical EV” and also resembles the phenotype of classical EV. Furthermore, atypical EV is associated with mutations in a number of genes involved in immune regulation (de Jong et al., 2018b). A number of studies have described a betaHPV DNA prevalence between 30–50% in SCC from immunocompetent persons (Harwood et al., 2006). In contrast, in immunosuppressed patients this number was observed to rise up to 90% (Harwood et al., 2000;Arnold and Hofbauer, 2012). As such, these retrospective epidemiological studies provided strong evidence that betaHPV may play a role in skin carcinogenesis, particularly in the immunosuppressed patient group (Bouwes Bavinck et al., 2008;Neale et al., 2013;Howley and Pfister, 2015). The prospective study conducted by the European EPI-HPV-UV-CA consortium further proved the strong association between high betaHPV DNA loads on the skin of immunosuppressed patients and the incidence rates of SCCs as they observed in the years post transplantation that OTRs showed higher total betaHPV DNA loads on their skin than what was observed in immunocompetent individuals. This is consistent with the hypothesis that higher viral DNA loads may also be paralleled by increased activity of betaHPV oncoproteins, which would explain the significantly elevated risk of SCC development seen in this patient group (Fig. 4) (Bouwes Bavinck et al., 2018).

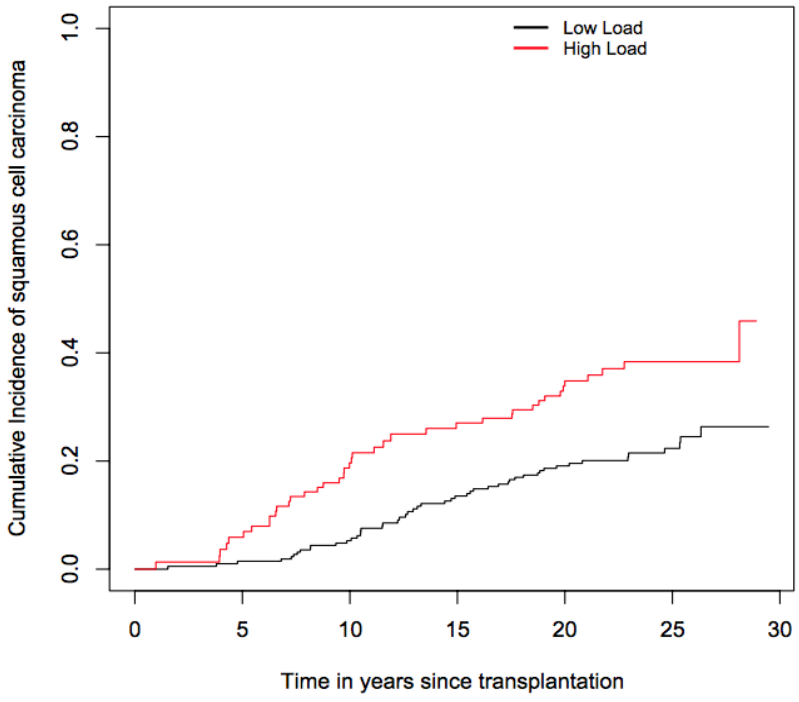

Fig. 4 Cumulative incidence of cutaneous squamous cell carcinoma in organ-transplant recipients. Epidemiological data provides strong evidence that infection with betaHPV is associated with the development of skin SCC (image taken from (Bouwes Bavinck et al., 2018)).

In the immunocompetent population the betaHPV DNA load in skin SCCs is lower than in immunosuppressed patients. The prevalence of betaHPV-DNA in precancerous AK (up to viral DNA 50 copies / cell) is higher than in SCCs (less than one viral DNA copy / cell), which is compatible with a carcinogenic role of betaHPV in early phases of skin cancer development (Pfister, 2003;Weissenborn et al., 2005;Schiller and Buck, 2011). Active betaHPV replication and early gene expression has been demonstrated in AKs as well as in the adjacent pathological epithelium of SCCs in OTRs, where betaHPV expression is also associated with increased expression of the cellular proliferation marker MCM7 (Borgogna et al., 2014). However, betaHPV DNA is barely found to be integrated into the DNA of skin cancer cells and is typically not transcriptionally active in the tumors of immunocompetent patients (Weissenborn et al., 2005). Thus, the presence of betaHPV is apparently not mandatory for maintenance of the malignant phenotype of SCCs in the general population. As betaHPV are also present in healthy skin of immunocompetent individuals and hair plucked out of their eyebrows, this might imply a connection between elevated risk of developing SCC, particularly in betaHPV-infected skin regions which are subjected to frequent UV exposure. In the majority of betaHPV infections of immunocompetent subjects the infection is usually controlled by the host immune system (Karagas et al., 2010).

Yet, betaHPV may be one risk factor raising the susceptibility to develop SCC. In the past it was shown that UV irradiation can directly cause such cancers in HPV8 positive cells (Akgül et al., 2005b). The current hypothesis in the field is that betaHPV may mediate tumorigenesis through a mechanism where betaHPV plays a co-factorial role in conjunction with UV-exposure in the initiation of skin cancer, a theory that is further underpinned by other studies (Hufbauer and Akgül, 2017;Viarisio et al., 2017;Viarisio et al., 2018). According to this “hit and run” hypothesis, betaHPV may act as a tumorigenic trigger that leaves behind oncogenic hits but becomes dispensable at later tumor stages when the production of virus DNA can no longer be maintained due to excessive tumor growth. This would also explain why betaHPV DNA is found in high copy numbers in precursor lesions such as AKs, while the virus itself appears to be dispensable for tumor maintenance in the general population (Doorbar et al., 2012;Heuser et al., 2016b;Hufbauer and Akgül, 2017;Hasche et al., 2018;Strickley et al., 2019). Why betaHPV is a part of the commensal skin flora at all is until now completely unknown. We do not know why humans are permitted to colonize the human skin, which raises the question if there may be a potential benefit for humans.

In this context, Strickley et al. (2019) studied the immunological effects in mice following mus musculus mouse papillomavirus 1 (MmuPV1) (Ingle et al., 2011) infection and subsequent challenge of the skin with a DMBA/TPA chemical carcinogenesis protocol. This approach represents a feasible murine model for cutaneous HPV infection, and the experiments were carried out with both immunocompromised and immunocompetent control mice. From their experiments the authors concluded that that skin PV may have a protective effect against genotoxic stress associated with skin cancer in MmuPV1-immune mice as this shielding effect was lost in immunocompromised mice. Transferred to humans this might mean that the betaHPV colonization of human skin of immunocompetent individuals may also confer similar protective effects. However, the very high viral DNA loads on the skin under immunosuppression could disrupt this protective shield, making such patients more susceptible towards the development of skin SCC (Strickley et al., 2019).

2.2.6 Studies of oncogenic functions of HPV8 early proteins in mice

The oncogenic and transforming abilities of betaHPV infections were further underpinned in transgenic mouse models. Our group created the very first HPV8 transgenic mouse line which encompassed the complete early genome region of HPV8 (CER, which contains the genes coding for E1/E2/E4/E6/E7) under the control of the keratin-14 (K14) promoter. These mice showed a vast number of papillomas, which was partially accompanied by moderate to severe dysplasia. In about six percent of the K14-HPV8-CER mice animals spontaneously developed SCCs without treatment or usage of any chemical carcinogenesis protocols (Schaper et al., 2005). Skin tumorigenesis in K14-HPV8-CER mice was accompanied by over-expression of matrix metalloproteases (MMPs) in epithelial as well as stromal fibroblasts (Akgül et al., 2006). UVA/B-irradiation and mechanical wounding of K14-HPV8-CER mouse skin led to prompt papilloma induction in about 3 weeks, which was always paralleled by an increased transgene expression. To study the role of individual HPV8 oncoproteins in tumor development in more detail, additional transgenic lines were created. Both HPV8-E6 (Marcuzzi et al., 2009) and HPV8-E2 (Pfefferle et al., 2008) have the ability to induce skin cancer independent from one another, albeit with distinct differences in kinetics and histology. The K14-HPV8-E6 mice represent a phenocopy of the K14-HPV8-CER mouse model. K14-HPV8-E2 transgenic mice also spontaneously developed infundibular hyperplasia, acanthosis as well as SCC.

The rate of tumor formation in E2 mice strongly depended on E2 mRNA expression. No spontaneous tumor development was observed in K14-HPV8-E7 mice over the course of 12 months. Of note, this mouse line had about 60 times less E7 mRNA expression than the K14-HPV8-CER mice. Yet, as soon as the K14-HPV8-E7 transgenic mice were exposed to chronic irradiation this led to a profound induction of hyperplasia and intra-epidermal abnormal keratinocytes, which also invaded the surrounding dermis (Heuser et al., 2016b). Thus, these various K14-HPV8 transgenic mouse models confirmed the oncogenicity of HPV8 early proteins *in vivo*.

2.2.7 The role of the E6 and E7 oncoproteins in tumorigenic processes

The primary factor regarding the oncogenic potential of both the E6 and E7 oncoproteins is predominantly defined by their “aggressiveness”, i.e. their ability to interfere with vital cellular processes whose interruption paves the way for carcinogenesis (Spriggs and Laimins, 2017). Both E6 and E7 interact with a large number of host target proteins (summarized in (Suarez and Trave, 2018)). *In vitro* molecular observations have demonstrated that the carcinogenic potential of betaHPV is mediated by prevention of host cell elimination following genotoxic stress such as UVB exposure (Hufbauer et al., 2015;Wendel and Wallace, 2017). In brief, the oncogenic effects of HPV result from a complex interplay of the viral E6 and E7 oncoproteins with various host proteins, which leads to HPV gaining control over cellular gene expression, inactivation of cellular proteins through direct binding, or targeted protein degradation by the host cell ubiquitin-proteasome (White and Howley, 2013;Galloway and Laimins, 2015;Hoppe-Seyler et al., 2018;Akgül et al., 2019). The HPV-E6 protein is cysteine-rich, basic and composed of approximately 150 amino acids which encompass two zinc-binding domains, which both contain two CXXC motifs conserved across all HPVs (Chen et al., 1998;Vande Pol et al., 1998). The significant oncogenic potential of the high-risk alphaHPV (e.g. HPV16) highly relies on the degradation of the tumor suppressor p53 by E6. HPV16-E6 binds to a LXXLL motif of the E6-associated protein (E6-AP), an E3 ubiquitin protein ligase, an event that ultimately leads to the degradation of p53 (Huh et al., 2007;Martinez-Zapien et al., 2016). Apart from p53 degradation, the E6/E6-AP complex is involved in the activation of the hTERT (human telomerase reverse transcriptase) gene (Klingelhutz et al., 1996). As a consequence, HPV16 infected cells display high telomerase activity. Some betaHPV E6 proteins can also induce hTERT, albeit with much lower E6-AP binding

affinity and thus betaHPV generally lack the ability to degrade p53 (Steger and Pfister, 1992;Bedard et al., 2008). Still, it was shown that, in contrast to alphaHPV, betaHPV-E6 proteins can interact with the LXXLL motif of the mastermind-like protein 1 (MAML1) (White and Howley, 2013). This betaHPV E6 exclusive ability results in a suppression of the Notch signaling pathway, which is of pivotal importance in promoting cell-cycle exit and differentiation (Brimer et al., 2012;Tan et al., 2012;White and Howley, 2013). Binding of betaHPV-E6 to MAML1 is therefore indispensable for the establishment of a productive HPV infection (Meyers et al., 2017). It is also known that the HPV8-E6 oncoprotein can modulate viral transcription via upregulation of the early and late viral promoters (Müller-Schiffmann et al., 2006). Having said that, the microRNA-203 (miR-203) levels are lower in EV-lesions positive for HPV8 as a consequence of E6 oncogene expression. In healthy cells, C/EBPα, a differentiation-regulating transcription factor and suppressor of UV-induced skin carcinogenesis, induces transcription of the miR-203 gene. In contrast, researchers observed that the C/EBPα/miR-203-pathway was impaired in HPV8-E6 positive cells, which resulted in an upregulation of the proliferation and stemness inducing transcription factor ΔNp63α, which coincidentally is also a target of miR-203 (Marthaler et al., 2017). Thus, targeting C/EBPα by betaHPV E6 appears to be a critical early step in betaHPV-mediated carcinogenesis in combination with UV light (Venuti et al., 2019). Furthermore, in our own group we could show that HPV8-E6 expressing cells fail to facilitate an effective response to UV-induced DNA lesions, which underpins the notion that betaHPV-E6 mediated suppression of DNA damage repair pathways is a critical step in HPV-driven skin carcinogenesis (Wendel and Wallace, 2017). Our group could show that HPV8-E6 has the ability to inhibit the UV-induced DNA damage repair by delaying the repair of thymine-dimers and DNA double stand breaks which had been caused by inhibition of key proteins in these pathways including the cell-cycle checkpoint regulator ATM, ATR and CHK1 (Hufbauer et al., 2015).

Coming to betaHPV-E7, in a similar fashion as mucosal high-risk alphaHPV types betaHPV E7 oncoproteins are comprised of approximately 100 amino acids, which can be compartmentalized into three conserved regions (CR): CR1 to CR3 (Tommasino, 2014). CR1 is required in cellular transformation processes while CR2 is necessary for cellular transformation and the degradation of pRb in high-risk HPV types for which it possesses a conserved L-X-C-X-E pRb binding motif and its related proteins p107 and p130 (White et al., 2012). The binding of High-risk alphaHPV E7 proteins to pRb, p107

and p130 has been extensively studied as all three proteins are strongly involved in cell cycle control through governing cellular gene expression (Tommasino, 2014;2017). High-risk alphaHPV target hypophosphorylated pRb which leads to its ubiquitin-proteosomal degradation whereas some betaHPV (such as HPV5 and HPV8) also bind to pRb, albeit with much lower affinity than high-risk alphaHPV, which results in its hyperphosphorylation (mediated by the cyclin-dependent kinases (cdk) cdk2 and cdk4/6) (Iftner et al., 1990;Yamashita et al., 1993;Caldeira et al., 2000;Caldeira et al., 2003;Akgül et al., 2007;Cornet et al., 2012). In healthy cells pRB suppresses genes associated with the E2F transcription factor through binding to E2F. In both cases the pRb/E2F complex is disrupted which has further downstream effects: The now free and active E2F transcription factors upregulate gene expression for proteins involved in G1/S phase transition. This leads to a deregulated G1/S phase during mitosis (White et al., 2012;Tommasino, 2017).

The CR3 region of both alphaHPV and betaHPV are located at the C-terminus and allow for interaction with various host cell complexes. This region also contains a zinc-binding domain which is comprised of two CXXC motifs, which are 29-30 amino acids apart (McLaughlin-Drubin and Munger, 2009). These two zinc-binding motifs are thought to be important for the assembly of homodimers and/or tetramers and protein stability. Furthermore, it has been shown for HPV8-E7 that it can relocate to the nucleus of infected keratinocytes. HPV8-E7 protein as it possesses a nuclear localization signal (NLS) for nuclear import (Onder and Moroianu, 2014) and additionally a nuclear export signal (NES) located in the zinc-binding domain (Onder et al., 2015). High-risk alphaHPV carry an additional casein kinase II (CKII) phosphorylation site in their CR2 domain (Firzlaff et al., 1989;Tommasino, 2017). Researchers could show that alphaHPV 6, 16 and 18 had such a recognition motif for CKII. It was observed that E7 was also targeted and subsequently phosphorylated by the kinase at serine residues within the binding motif for CKII (Firzlaff et al., 1989). Furthermore, these studies revealed that phosphorylation of HPV6-E7 (coined HPV6b-E7) resulted in E7 binding to pRb. Secondly, if the serine residues of the CKII recognition site of E7 were removed this led to a stark decrease in its transforming capabilities in HPV6- as well as HPV16-E7 positive cells. In contrast to these findings the E7 protein of betaHPV type 8 did not seem to be phosphorylated. A reason for the lack of HPV8-E7 phosphorylation was provided by the observation that the amino acid sequence of HPV8-E7 protein only possessed a crippled CKII binding motif.

In vitro experiments had already shown that these truncated CKII sites resulted in a much lower phosphorylation ratio of HPV8-E7 by CKII (Iftner et al., 1990). It is broadly accepted that the HPV E7 oncoprotein allows for cell cycle progression of infected keratinocytes even after having sustained significant DNA damage, which would normally result in cell cycle arrest for DNA damage repair (DDR) pathways or apoptosis if the cell cannot be salvaged (Tommasino, 2017).Unsurprisingly, interfering with these mechanisms results in genomic destabilization which consequently then leads to an accumulation of DNA damage (Malumbres and Barbacid, 2009;Wendel and Wallace, 2017). Another target of HPV, p53, is essential to avoid aberrant cell proliferative processes under genotoxic stress. It targets transcriptional factors of several pathways, which are involved in DNA-damage tolerance and apoptosis, among others (Yu and Zhang, 2005;Beckerman and Prives, 2010).

It is still poorly understood how high-risk alphaHPV E7 abrogates p53 activity, but more data is available regarding the mechanism of betaHPV E7 proteins by which they inhibit the indispensable tumor suppressor p53 through upregulation of its antagonist ΔNp73 (located on the gene for p73, which encodes one of two isoforms: a) TAp73, which is pro-apoptotic and b) ΔNp73m which has anti-apoptotic anti-apoptotic properties (Lai et al., 2014)), whose activity results in a negative feedback loop with p53 (Bailey et al., 2011). BetaHPV-38 activity enhances accumulation and also stabilizes ΔNp73 both on its transcriptional and post translational level (Saidj et al., 2013;Tommasino, 2017). HPV38 E6 and E7 carry out a double phosphorylation of p53 at serine 15 and 392 which contribute to increased ΔNp73α expression and facilitate the translocation of IκB kinase to the nucleus. In addition, beta HPV38 E7 promotes, via an unknown mechanism, nuclear translocation of the IκB kinase beta (Tommasino, 2017), which stabilizes ΔNp73α through phosphorylation at serine 422 that (Accardi et al., 2011). Together with DNMT1 and EZH2 both proteins also form a transcriptional regulatory complex which binds to p53 controlled promoters and thus disrupts DNA damage repair and apoptotic pathways. This could mean that the HPV38-mediated inhibition of p53 also influence DNA damage related genes that are dependent on p53 activity (Fischer, 2017;Tommasino, 2017;Wendel and Wallace, 2017). Apart from the very well described interaction of HPV8-E7 with pRb, different studies and experimental approaches led to the identification of a nuclear, cytoplasmic as well as mitochondrial interaction partners which are summarized in table 2.

HPV8-E7 interaction partners	Localization	Citation
C/EBP	cytoplasmic	(Sperling et al., 2012)
C/EBPbeta	cytoplasmic	(Sperling et al., 2012)
CNP	cytoplasmic	(Grace and Munger, 2017)
COPA	cytoplasmic	(Grace and Munger, 2017)
E2F	cytoplasmic, nuclear	(White et al., 2012;White and Howley, 2013)
ELF1	nuclear	(Rozenblatt-Rosen et al., 2012)
ERG	cytoplasmic, nuclear	(Rozenblatt-Rosen et al., 2012)
KCMF1	cytoplasmic	(White et al., 2012;White and Howley, 2013;Grace and Munger, 2017)
LOX	cytoplasmic	(Grace and Munger, 2017)
MAP2	cytoplasmic	(White et al., 2012;White and Howley, 2013)
MAP4	cytoplasmic	(Grace and Munger, 2017)
Myo-1C	cytoplasmic, nuclear	(Oswald et al., 2017)
P600	nuclear	(White et al., 2012)
PDLIM7	cytoplasmic	(White et al., 2012;White and Howley, 2013;Grace and Munger, 2017)
RB1	nuclear	(White et al., 2012;White and Howley, 2013;Grace and Munger, 2017)
RBL1	nuclear	(Grace and Munger, 2017)
RBL2	nuclear	(White et al., 2012) (White and Howley, 2013)
PTPN14	cytoplasmic, nuclear	(White et al., 2012); White and Howley, 2013; (Grace and Munger, 2017)
PTPN21	cytoplasmic	(Grace and Munger, 2017)
PTRF	cytoplasmic, nuclear, mitochondrial	(Rozenblatt-Rosen et al., 2012;White et al., 2012)
REL	nuclear	(Rozenblatt-Rosen et al., 2012)
SQRDL	mitochondrial	(Grace and Munger, 2017)
TAF110	mitochondrial	(Enzenauer et al., 1998)
TBP	cytoplasmic, nuclear	(White et al., 2012) (White and Howley, 2013)
UBR4	cytoplasmic, nuclear	(Grace and Munger, 2017)

VKORC1	cytoplasmic (endoplasmic reticulum)	(Rozenblatt-Rosen et al., 2012;White et al., 2012)
WWC2	cytoplasmic	(Grace and Munger, 2017)
POLR1C	nuclear	(Rozenblatt-Rosen et al., 2012)
MEOX2	nuclear	(Rozenblatt-Rosen et al., 2012)
WDYHV1	cytoplasmic, nuclear	(Rozenblatt-Rosen et al., 2012)

Table 2: Known HPV8-E7 interaction partners described in literature.

Directed analyses of E7 interaction partners have demonstrated that betaHPV8-E7, 25 and 92 have the ability to interact with the tumor suppressor PTPN14, which is a tyrosine phosphatase and is also involved in cell-cell adhesion, cell-matrix adhesion, cell migration, cell growth and also regulates TGF-beta gene expression, and as such modulates epithelial-mesenchymal transition. Consequently, many high and low-risk alphaHPV E7 target and degrade PTPN14 to maintain proliferation of infected cells (White et al., 2016;Szalmas et al., 2017). However, the exact impact of the interaction between betaHPV E7 and PTPN14 is still being researched. The protein is required in the Hippo signaling pathway as it facilitates the translocation from the nucleus to the cytoplasm of the YAP1-Hippo pathway transcription factor. (Wang et al., 2012). This observation implicates that betaHPV E7 is involved in interfering with the Hippo pathway, which might enable betaHPV E7 to destabilize PTPN14. Coincidentally, this pathway is also crucial for controlling keratinocyte proliferation and apoptosis (Ganem et al., 2014). It has been shown in the past that phosphorylation of Yap1 plays a pivotal role in induction of apoptosis in response to DNA damage (Levy et al., 2008). As betaHPV infect cutaneous keratinocytes of the skin and infected cells at sun exposed sites are constantly subjected to UV light irradiation – which represents an environment where cell cycle arrest and apoptotic processes occur more frequently due excessive DNA damage – it is a compelling thought that betaHPV E7 may be able to interfere with these processes in infected cells to complete their viral life cycle. However, the uncoupling of cell cycle progression from checkpoint control mechanisms in combination with increasing amounts of DNA damage may then ultimately lead to aberrant cell behavior, including hyperproliferation (Heuser et al., 2016b). HPV8-E7 is also seemingly able to alter the cellular proteome. In the past, quantitative 2D gene expression (DiGE) experiments coupled with mass spectrometry had resulted in the identification of actin binding proteins which are modulated in an HPV8-E7 dependent

manner (Akgül et al., 2009). Our group also recently showed that expression of the HPV8-E7 oncoprotein alters β-catenin and zona occludens-1 proteins which results in malfunctioning tight junctions and cell adherence leading to a reorganization of tissue coherence and epithelial mesenchymal transition (EMT) which is akin to a more stem-cell like de-differentiation in HPV8-E7 positive keratinocytes (Chen et al., 2016;Heuser et al., 2016a). Previous research of our group had already shown that HPV8-E7 plays a pivotal role in inducing and regulating keratinocyte proliferation and invasion. E7 expressing keratinocytes grown as organotypic skin cultures (OSCs) based on a de-epidermalized human dermis as a scaffold displayed hyperproliferation and were positive for both cyclin E and p16INK4a indicating that E7 is able to overcome p16INK4a induced cell cycle arrest (Westphal et al., 2009). In addition, HPV8-E7 positive keratinocytes lost their normal polarity and invaded through the underlying dermis (Akgül et al., 2005a). Downward migration of the keratinocytes into the dermis was facilitated by the degradation of basement membrane components collagen VII, collagen IV and laminin-332, which was paralleled by expression of matrix metalloproteinases (MMP)-1, MMP-8, and membrane-type (MT)1-MMP (Akgül et al., 2005a;Smola-Hess et al., 2005).

However, when organotypic cultures were alternatively composed of a collagen type I gel as matrix, no invasive phenotype of betaHPV early protein expressing cells was observed (Boxman et al., 2001). This hypothesis is further supported by the observation that in HPV8-E7 positive cells an upregulation of metalloproteases in the ECM was observed, which leads to a change of its composition that in turn promotes pathological cell migration and invasion in these cells (Akgül et al., 2006;Hasche et al., 2018). HPV8-E7 cells do not only upregulate fibronectin expression (which is another hallmark feature of EMT), but also secrete the protein into the ECM micro-environment, which curiously stimulates fibroblasts in the basement membrane to also produce more fibronectin. This further enhances invasive behavior of HPV8-E7 positive keratinocytes. Keratinocytes grown on fibronectin simultaneously also showed an over-representation of the integrin α3β1 transmembrane complex on the cell surface and an increased tendency for invasive keratinocyte behavior (Heuser et al., 2016b). Therefore, the interaction between the integrin α3β1 and particularly the ECM protein fibronectin seems to be of pivotal importance in the development of skin carcinomas and indicates that the HPV8-E7 protein is heavily involved in this process (Heuser et al., 2016b).

2.3 Thesis Aims

The overall aim of this thesis was to arrive at a deeper understanding of how HPV8-E7 reprograms and hijacks the cell, thereby interferes with vital cellular mechanisms and how the interference with host cellular mechanisms may ultimately contribute to malignancies such as cancer. To this end we used different experimental approaches such as transcriptomics, proteomics / phospho-proteomics as well as protein-protein interaction studies to identify novel cellular targets of HPV8-E7 (as shown in Fig. 5). Afterwards identified targets were to be confirmed *in vivo* in organotypic skin cultures, in the skin of transgenic mice as well as human patient material.

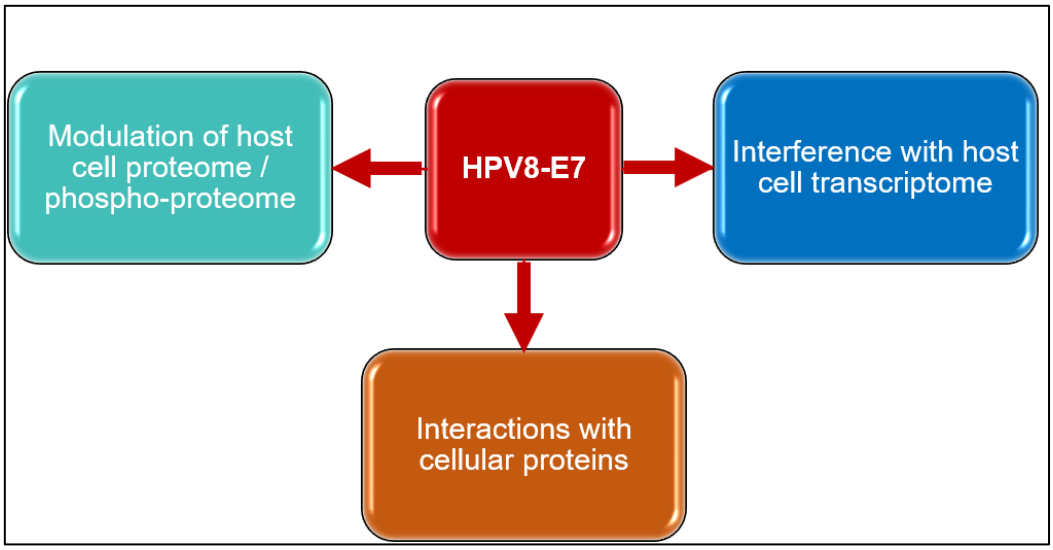

Fig. 5 Aims of this thesis. Experimental approaches to unravel novel oncogenic activities of HPV8-E7.

III. Results

3.1 Virology. 2019; 535:136-143

HPV8 activates cellular gene expression mainly through Sp1/3 binding sites. **Kirschberg M***, Heuser S*, Syed AS, Steger G, Majewski S, Hufbauer M, Akgül B. *Equal contribution.

Own substantial contributions to this publication:

- Analyzed and interpreted the data shown in Fig. 1 and Fig. 2;
- Performed immunofluorescence stainings shown in Fig. 3;
- Performed Western Blots and RT-qPCRs shown in Fig. 5;
- Interpreted all data
- Wrote the manuscript and revised the paper in response to reviewer comments.

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HPV8 activates cellular gene expression mainly through Sp1/3 binding sites

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ABSTRACT

The human papillomavirus type 8 (HPV8) is associated with skin cancer development. The goal of this study was to investigate the effects of HPV8 oncoproteins on cellular gene expression and the identification of key regulators. We performed affymetrix microarray analyses to identify differentially expressed genes and common sequence motifs and identified Sp1/3 binding sites as being crucial. In transient transfection assays, we confirmed that HPV8-E7 stimulates the activity of Sp1/3 promoters. Interestingly, the HPV8-E7^{L23A} mutant, which cannot trigger keratinocyte invasion was unable to activate fibronectin gene expression. In skin models or HPV8 positive skin cancers we found a peculiar deposition of fibronectin in the dermal compartment, and a correlation of Sp3 and fibronectin in the nucleus of HPV8-positive keratinocytes.

Taken together, we identified that HPV8-E7 exerts control over cellular gene expression through Sp1/3 binding motifs, which may contribute to HPV8-mediated keratinocyte transformation and subsequent fibronectin-dependent invasion.

1. Introduction

Epidemiological and experimental data demonstrate an involvement of human papillomaviruses of genus betapapillomavirus (betaHPV) in the development of cutaneous squamous cell carcinoma (SCC) (Howley and Pfister, 2015; Tommasino, 2019). In particular, Epidermodysplasia verruciformis (EV) patients suffer from betaHPV mediated skin carcinogenesis. EV is a rare autosomal recessive hereditary skin disease characterized by increased susceptibility for betaHPV infection, such as HPV8 (Imahorn et al., 2017). It is also an accepted fact, that there is a direct link between betaHPV infection and cancer development in immunosuppressed organ-transplant recipients (OTR) (Bouwes Bavinck et al., 2018).

To further our understanding regarding the oncogenic potential of HPV8, we had previously generated a variety of HPV8 transgenic mouse models, the first of which is expressing the complete early genome region (CER) under the control of the keratin-14 promoter (K14). These mice displayed formation of skin papillomas with varying degrees of dysplasia as well as skin SCCs (Hufbauer et al., 2010; Schaper et al., 2005). K14-HPV8-E7 mice did not show papilloma formation, but exhibited carcinoma development (Heuser et al., 2016b).

Using monolayer cultures of primary human adult keratinocytes (PHK) the expression of HPV8-CER and HPV8-E7 induced an abnormal keratin expression pattern, that included simple epithelial (K8, K18, K19), hyperproliferation-specific (K6, K16), basal-specific (K14, K15) and differentiation-specific (K1, K10) keratins. The expression of hyperproliferation-associated keratins in HPV8-E7 cells was also paralleled by loss of G1/S control and cells were able to overcome the mitotic checkpoint (Akgül et al., 2007). Furthermore, we also demonstrated in the past that expression of HPV8-E7 is critical for the induction of keratinocyte invasion in organotypic skin cultures (OSC) (Akgül et al., 2005; Westphal et al., 2009). More recently, we could show that HPV8-E7 mediated keratinocyte invasion is triggered by the extracellular matrix protein fibronectin (FN), and an increase of integrin α3β1 surface presence. Intriguingly, we detected depositions of FN in adjacent tumoral stroma of HPV8-positive skin SCCs (Heuser et al., 2016b). Further evidence for the existence of the α3β1/FN axis arose from the observation that the HPV8-E7 mutant L23A (HPV8-E7^{L23A}) was neither capable of affecting α3β1 surface presence nor was it able to trigger keratinocyte invasion (Heuser et al., 2016b; Hufbauer and Akgül, 2017).

In this study, we aimed at a deeper understanding regarding the mechanisms involved in HPV8 mediated gene regulation.

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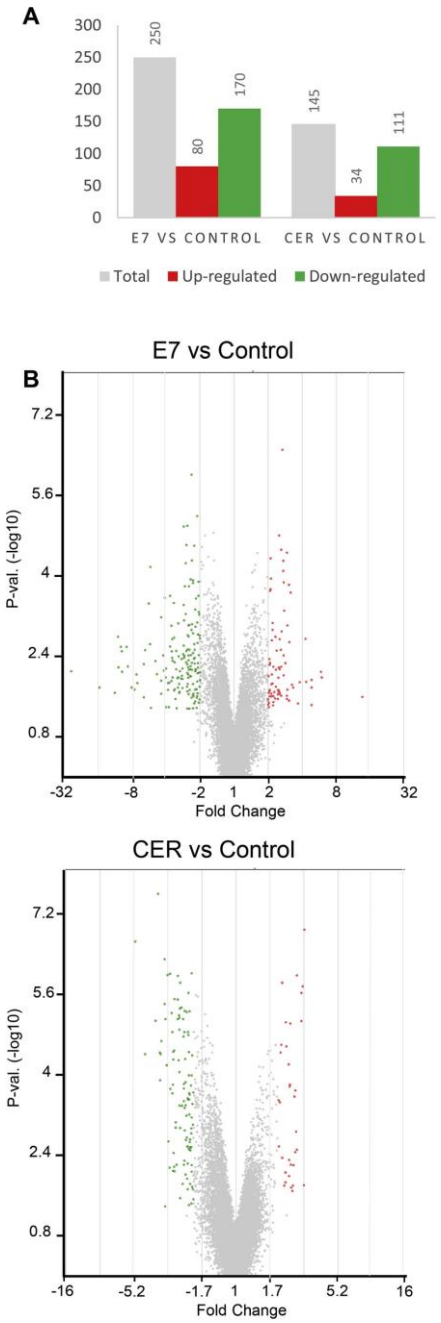

Fig. 1. Global gene expression profiling. A) Bar charts showing differentially expressed genes in HPV8-E7 vs. control or HPV8-CER vs. control (grey: total number of genes; red: up-regulated genes; green: down-regulated genes. B) Volcano plot depicting fold-change gene expression in HPV8-E7 vs control or HPV8-CER vs. control. (red: up-regulated genes; green: down-regulated genes).

higher than in CER (Fig. 1A). Furthermore, the overall fold changes in gene expression were also stronger in the HPV8-E7 versus control analysis as seen in the Vulcan plots (Fig. 1B). The gene ontology analysis sub-categorizing differentially genes in biological processes, cellular components and molecular functions are shown in Supplementary Fig. 2 and Fig. 3.

In order to understand how HPV8 might exert control over cellular gene expression, less stringent gene lists were translated to the Human9999 genome, which contained the first 9999bp of the upstream regions of all genes present in the ENSEMBL database. Regulatory sequences of five to eight base pairs between 10 and 500 bases upstream of dysregulated genes were matched with a $p < 0.05$ threshold. Afterwards, search criteria were further narrowed down to conserved regulatory sequences of 5–8 bp in length between 10 and 500 bp upstream of each of 295 picked genes, requesting exact matches with $p < 0.05$. This motif enrichment analysis led to the identification of four nucleotide sequences with 6 bp: 5′-CGCCCC-3′, 5′-CCGCCT-3′, 5′-CGCCTC-3′, 5′-TCCGCC-3' (Fig. 2A). Interestingly, sequences 1 to 3 are complementary to the decanucleotide consensus sequence of the transcription factors Sp1/3: 5'-(G/T)GGGCGG(G/A) (G/A) (C/T)-3' (Li and Davie, 2010; Nagaoka et al., 2001). The reverse complement orientation of sequence 4 also overlapped with the consensus Sp1/3 binding motif (Fig. 2B). The analysis of the microarray data led to the identification of only one Sp-family member, namely Sp3 as being 2.5-fold upregulated in HPV8-E7 expressing PHK. To confirm if HPV8-E7 has the ability to trans-activate promoters containing Sp1/3 binding motifs, we tested its activity on the synthetic pALuc-Sp1/3 promoter construct, which contains two Sp1/3 binding sites in front of a TATA box, as well as the luciferase gene (Fig. 2C). PHK were transiently co-transfected with pALuc-Sp1/3 and expression vectors coding for HPV8-E7. Luciferase activity of the promoter construct, measured 48 h post-transfection, was increased up to 8-fold in the presence of E7 (Fig. 2D). These results support our hypothesis that HPV8-E7 may exert control over cellular gene expression through Sp1/3 binding sites.

3.2. Expression of Sp3 and fibronectin correlates in HPV8 positive epithelia

Based on our own research we already know that FN expression is indeed modulated by HPV8-E7 (Heuser et al., 2016b) and is crucial for the invasive phenotype of E7 positive cells. To test whether Sp3 may be involved in the control of FN expression in betaHPV-positive differentiating epithelia, we first analysed the staining patterns in OSCs comprised either of PHK harboring the empty vector pLXSN, or PHK expressing the HPV8-E7. In epithelium repopulated with the control keratinocytes we only detected very weak Sp3 levels in the differentiating keratinocytes, and FN was only found, as expected, in the dermal compartment of the OSCs. Interestingly, compared to the control, Sp3 signals were more pronounced in the HPV8-E7 OSCs. Even more exciting though was the observation, that in the HPV8-E7 OSCs we observed strong stromal FN deposition as well as nuclear FN, which also partially correlated with Sp3 signals (Fig. 3A). In skin lesions from EV patients we observed an even more pronounced correlation in respect to the Sp3/FN staining patterns observed in the OSCs. In the EV lesions, a vast majority of keratinocytes in all epithelial layers were found to be expressing Sp3 as well as nuclear FN (Fig. 3B), which was in stark contrast to normal/healthy skin of non-EV patients, where we could not detect any FN deposition in the epidermal compartment at all. Interestingly, when staining skin sections from the same OSCs and EV tissues for Sp1 with several antibodies we did not observe any Sp1 signal anywhere throughout the skin (data not shown).

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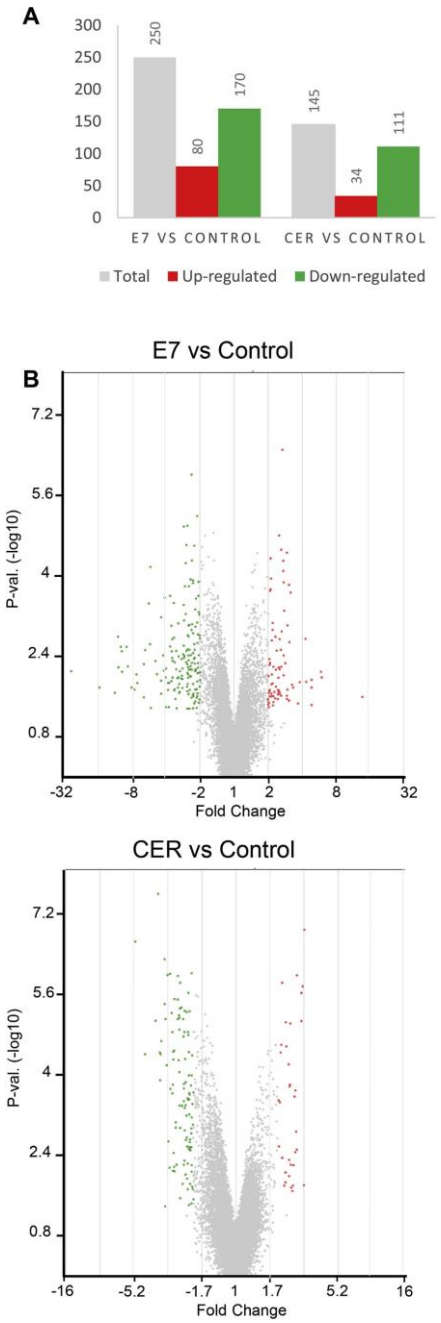
We next investigated the mechanism by which HPV8-E7 may exert control over FN expression. It is known that the human FN promoter



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A

No.	Sequence	observed	p-value	random rate	observed rate	single p	tests
1	CGCCCC	135/295	0.00375	25.22%	31.54%	$2.3x10^{-7}$	16384
2	CCGCCT	104/295	0.0417	18.74%	23.34%	$2.6x10^{-6}$	16384
3	CGCCTC	106/295	0.00339	18.74%	22.75%	$2.07x10^{-7}$	16384
4	TCCGCC	98/295	0.000594	18.74%	19.74%	$3.62x10^{-8}$	16384

B

```
5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3'     Sp1/3 Consensus Binding Site
3'-(C/A)CCCGCC(C/T)(C/T)(G/A)-5'
          CGCC C    C                seq. 1
         CCGCC   T                   seq. 2
          CGCC   T  C                seq. 3
         CCGCC   T                   seq. 4
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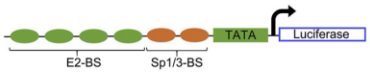
C



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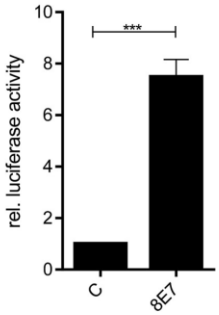

Fig. 2. Identification of regulatory sequences in HPV8-E7 regulated genes using upstream sequence analysis. (A) Sequence: Identified conserved sequence motif; Observed: number of genes in which the conserved motif was found; Random rate: % of genes (intrinsic probability) expected to contain the nucleotide combination if the nucleotide sequences were strictly random; Observed rate: the probability of the observed sequence occurring relative to the genes not regulated by HPV8; Single p: The probability that a particular sequence would be found, if only one test had been performed; Tests: number of oligomers tested having a length of the found sequence motif). (B) Sequence alignments of Seq. 1–4 (identified in A) with the Sp1/3 consensus binding site. (C) Schematic representation of a synthetic luciferase reporter construct composed of the minimal adenovirus major late promoter (MLP) in front of four E2 and two Sp1/3 binding sites. (D) The luciferase construct was transfected into PHK with HPV8-E7 expression vectors. Increased luciferase levels were measured in presence of HPV8-E7. Expression level of the corresponding co-transfected empty vector (control) was set as 1 (n = 3).

contains a Sp1/3 binding site at position (−136 to −127 (Michaelson et al., 2002) (Fig. 4A). To confirm that HPV8-E7 is capable of inducing the FN promoter, and in order to narrow down where the responsive element within the promoter region is, luciferase promoter assays were performed with FN promoter constructs pFN(1.2 kb)-LUC, or the truncated promoter constructs pFN(0.5 kb)-LUC, and pFN(0.2 kb)-LUC, respectively. PHKs were transiently transfected with the FN promoter constructs together with HPV8-E7 expression vector or empty vector control. HPV8-E7 activated all three tested FN promoter constructs about 2-fold (Fig. 4B). To confirm that the Sp1/3 binding site plays a pivotal role in FN promoter regulation in the presence of HPV8-E7, we generated a variant of the pFN(0.2 kb)-LUC with a mutated Sp1/3 binding motif (pFN(0.2 kb)-LUC-Sp1/3mut). This mutant failed to activate the FN promoter in the presence of HPV8-E7 as shown in Fig. 4B, indicating that the HPV8-E7 protein may exert control over Sp3 to enhance FN expression.

3.4. The invasion-deficient mutant HPV8-E7-L23A is incapable of transactivating the fibronectin promoter

To address the question whether the HPV8-E7^{L23A} mutant may be defective in stimulating the FN promoter, the pFN(0.2 kb)-LUC and the pFN(0.2 kb)-LUC-Sp1/3mut constructs were co-transfected with expression vectors coding for HPV8-E7^{wt} or -E7^{L23A} into keratinocytes. As expected, in C33A and PHKs, HPV8-E7^{wt} activated the pFN(0.2 kb)-LUC construct, whereas the HPV8-E7^{L23A} mutant, which is known to be as stable as the wildtype E7 protein (Heuser et al., 2016b), failed to activate both pFN(0.2 kb)-LUC and pFN(0.2 kb)LUC-Sp1/3mut (Fig. 5A and B). Furthermore, in subsequent Western blots we could show that Sp3 protein levels were strongly increased in HPV8-E7^{wt} expressing cells, whereas in HPV8-E7^{L23A} positive cells there was a visible reduction Sp3 levels were less pronounced than in E7^{WT} cells (Fig. 5C). The HPV8-E7^{wt} dependent increase of Sp3 appears to be achieved through transcriptional activation, as Sp3 mRNA levels were significantly elevated in HPV8-E7^{wt} cells compared to the control (Fig. 5C, ***p = 0.001). In addition, the invasion deficient HPV8-E7L23A mutant was less able to trigger Sp3 mRNA expression than HPV8-E7^{WT} (Fig. 5D, *p = 0.0415). Unexpectedly, as neither the microarray data nor immunofluorescence tissue staining had shown Sp1 as a significant target, Sp1 protein levels were also found to be elevated in both HPV8-E7wt and HPV8-E7L23A PHK compared to the control (Fig. 5D). However, we observed that Sp1 fluctuates between different keratinocyte cell lines expressing HPV8-E7 (data not shown).

4. Discussion

In this study, we explored the effects of HPV8 early proteins on cellular gene expression. Differentially expressed genes were identified by microarray analyses of RNA from PHK expressing either HPV8-E7 alone or all early proteins simultaneously. By comparing these two populations we saw an about two-fold enrichment of upregulated genes in E7 cells, indicating that the co-expression of E7 with E1^E4, E2, E6 and E8^E2 may affect the transcriptional outcome in oncogene positive cells.

The main objective of this study, however, was the identification of upstream regulators hijacked by HVP8 to manipulate cellular gene expression. For this purpose, we used the generated microarray data, and identified the transcription Sp3 as a key regulator of cellular genes in HPV8 positive PHK. Sp3 belongs to a transcription factor family with four known members, namely: Sp1, Sp2, Sp3 and Sp4. Despite being one transcription factor family, there is only very little functional overlap amongst the Sp proteins, with the exception of Sp1 and Sp3, which play a dual role in the regulation of gene expression through Sp1/Sp3 binding sites (Huang et al., 2015). Both Sp1 and Sp3 together control more than 12.000 genes, and are therefore often collectively referred to as Sp1/3 (Li and Davie, 2010). Depending on the binding

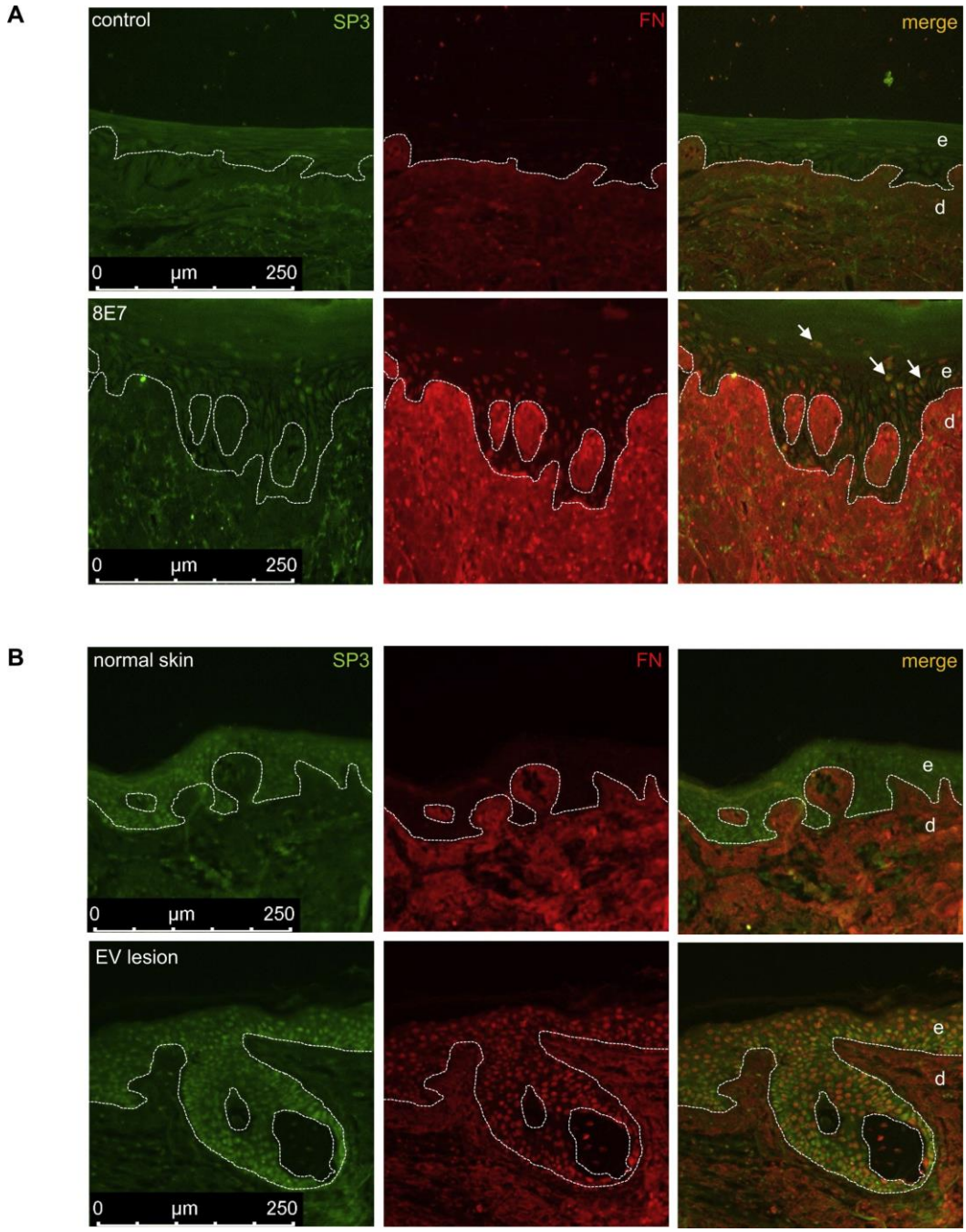

Fig. 3. Sp3 and FN expression patterns in HPV8 positive skin compared to control. (A) Representative immunofluorescence staining of Sp3 and FN in OSCs, which were repopulated with PHK harboring the empty retroviral vector pLXSN (control) or pLXSN-8E7, respectively, grown for 14 days at the air-liquid interphase (n = 3). (B) Representative immunofluorescence staining of Sp3 and FN in healthy skin and skin SCCs from EV patients positive for HPV5, 8, 20, 23, 36, 50 (green: Sp3, red: FN; dashed line: basement-membrane zone; d: dermis; e: epidermis; white arrows: cells double-positive for Sp3 and FN).

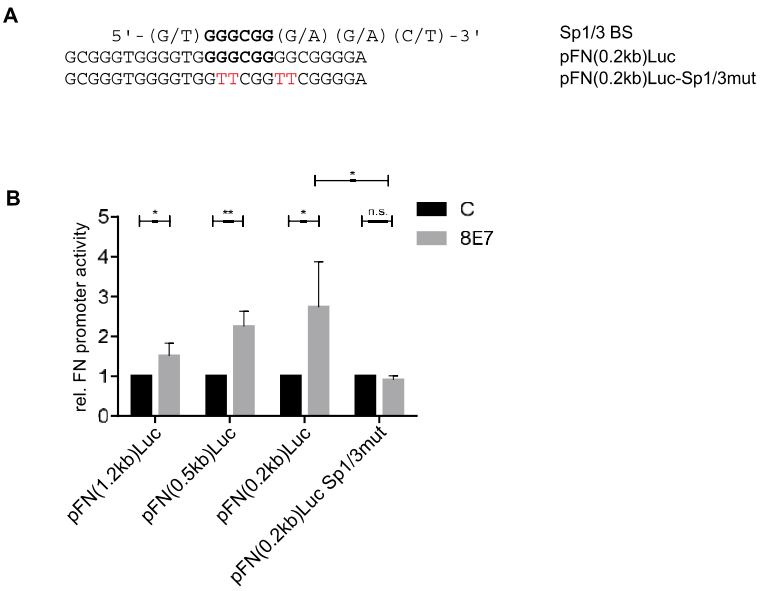

Fig. 4. The fibronectin promoter activity is increased by HPV8-E7. (A) Schematic presentation of putative transcription factor binding sequence within the 200 bp fragment of the human FN promoter. The sequence alignment shows the consensus Sp1/3 binding site and the mutation strategy for the Sp1/3 binding site (highlighted in red). (B) FN promoter constructs were transfected into C33A-keratinocytes with pLXSN-8E7 expression vectors. The expression level of the corresponding co-transfected empty pLXSN vector (control) was set as 1. FN: FN (n = 3). (C) FN promoter activation by HPV8-E7 is dependent on a functional Sp1/3 binding site.

region within a given promoter or Sp1/3 relative ratios, Sp3 may act as either an activator or repressor of gene expression. There is also growing evidence that Sp proteins play a critical role in tumorigenesis and metastatic potential in many tumour types by regulating expression of cell cycle genes, expression of the vascular endothelial growth factor, tumorigenesis, and apoptotic processes (Mertens-Talcott et al., 2007). Taken together, their transcriptional activity depend on a) the cell type b) the DNA binding site and finally c) Sp1/3 relative ratios. Considering

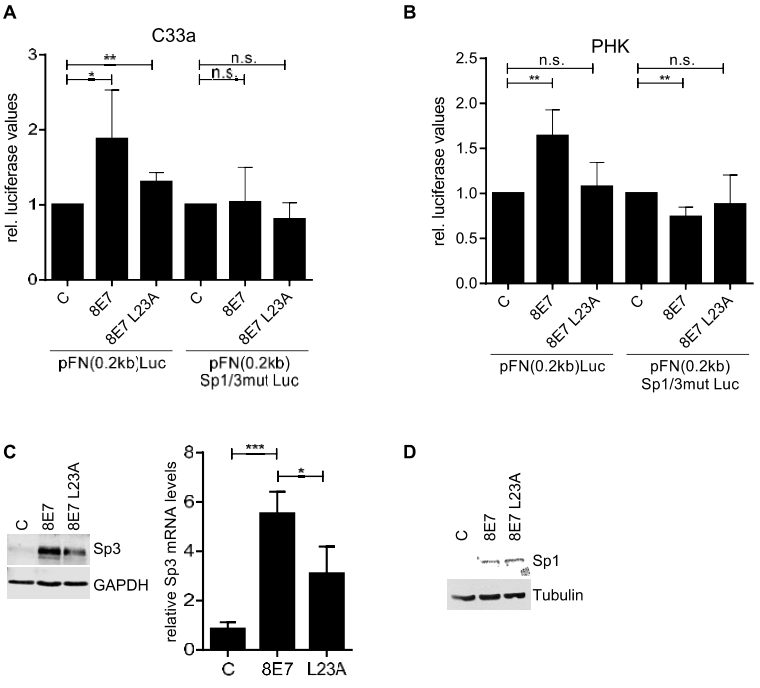

Fig. 5. HPV8-E7^{L23A} lacks the ability to activate the FN promoter. (A, B) pFN(0.2 kb)LUC and pFN (0.2 kb)LUC-Sp1/3mut constructs were transfected into C33A and PHK together with pLXSN-8E7^{wt}, pLXSN-8E7^{L23A} or control vector and luciferase was measured (n = 3). (C) Representative immunoblot of RIPA extracts from PHK expressing HPV8-E7^{wt} or HPV8-E7^{L23A} tested for Sp3 protein expression (n = 3). Equal loading was confirmed by immunoblotting for GAPDH. Relative mRNA levels of Sp3 in PHK (n = 3) expressing either E7^{WT} or E7^{L23A}. Sp3 mRNA levels were normalized to the HPRT1 mRNA levels. The relative ratio of the control was set to 1. (D) Representative immunoblot of RIPA extracts from PHK expressing HPV8-E7^{wt} or HPV8-E7^{L23A} tested for Sp1 protein expression (n = 3). Equal loading was confirmed by immunoblotting for tubulin.

that we were only able to detect Sp3, but not Sp1 in the epidermal layers of HPV8 positive skin of EV patients and HPV8-E7 positive OSCs suggests that HPV8 may alter Sp1/Sp3 relative ratios.

When grown on FN, HPV8-E7 expressing keratinocytes undergo epithelial-mesenchymal transition (EMT), a process that is accompanied by a downregulation of E-cadherin and upregulation of N-cadherin, which enhances both cell motility and thus also invasion (Heuser et al., 2016b). FN production did not only result from E7 expression in keratinocytes, but also from stimulated fibroblasts in monolayer cultures (Heuser et al., 2016b). These previous observations highlighted the importance of cell-matrix interactions, especially the involvement of FN in betaHPV induced keratinocyte transformation. Utilizing FN promoter truncation mutants, we could also show that HPV8-E7 not only activates the FN promoter, but also that the HPV8-E7 responsive element is most likely located within the 0.2 kb upstream promoter region. This particular fragment still showed luciferase activity comparable to pFN(1.2 kb)-LUC in the presence of HPV8-E7 (Fig. 4B). By mutating the Sp1/3 binding site within this region and testing for HPV8-E7-mediated activation, we could prove that HPV8-E7 mediates FN promoter activation through the Sp1/3 binding motif (Fig. 4B). Since we could demonstrate that HPV8-E7^{wt} upregulates Sp3 and Sp1 protein levels in monolayer cultures of PHKs we hypothesize that E7 mediated changes of Sp1/3 ratios may be the crucial underlying regulatory mechanism for the control of cellular gene expression. Since the HPV8-E7^{L23A} was less proficient at inducing Sp3 and FN expression (Fig. 5), we speculate that Sp3 may be involved in controlling FN dependent processes regulating keratinocyte invasion.

In addition, we could show in OSC, that FN is deposited in the dermal compartment beneath HPV8-E7 positive epithelial layers. In addition, nuclear FN was found in these HPV8-E7 positive keratinocytes and more strikingly, staining patterns of both Sp3 and nuclear FN were found to be correlating (Fig. 3). This effect was even more pronounced in EV skin SCCs, which was in stark contrast compared to the FN/Sp3 distribution patterns found in healthy skin, where we observed no FN deposition in the epidermal compartment. Our results provide compelling data that hint at a previously unknown regulation of the transcription factor Sp1/3 by HPV8-E7, which may be the driving element behind the observed FN over-expression in HPV8-E7 positive keratinocytes.

It was reported decades ago that cellular FN apparently can be part of the nuclear matrix of cancer cells. In hepatocellular carcinoma cells FN was even found in the nuclear matrix of cells cultivated in FN deprived medium and that it was even preferentially associated with the nuclear matrix (Jagirdar et al., 1985). Interestingly, such nuclear deposition has also been described in HeLa cells. In cervical carcinomas FN staining patterns were described as diffuse (Goldberg et al., 1998), which does not exclude the possibility that nuclear FN may indeed also be found in cervical cancers (Zerlauth et al., 1988). In that light, it is a quite intriguing finding that we now show enhanced levels of nuclear FN and a correlation with over-expression of the transcription factor Sp3 in betaHPV positive skin SCC (Fig. 3). Regarding the modulation of FN expression by other HPV it has previously been described that both the HPV16-E6 and -E7 proteins are able to promote FN expression in monolayer cultures of primary keratinocytes (Hellner et al., 2009). Interestingly, in our study FN was also found to be deposited in the tumor stroma but also in the nucleus of betaHPV positive skin cancers from non-EV patients (Heuser et al., 2016b) as well as skin lesions from EV patients as shown in Fig. 3B. These observations are of particular relevance in respect to immunosuppressed individuals such as organ transplant recipients, who frequently suffer from high betaHPV loads in their skin as well as a greater susceptibility to develop skin tumours (Bouwes Bavinck et al., 2018; Dell'Oste et al., 2009).

While it has been known that extracellular FN regulates keratinocyte homeostasis, it is completely unknown, whether the presence of nuclear FN may be a result of changes to the FN molecule itself – altering its' binding affinities – or if it is caused by alterations of the tumour nuclear matrix. It is also entirely unknown whether nuclear FN expression might influence skin cell motile behavior. Taken together, our results warrant further studies to confirm the clinical importance of FN deregulation for wound healing and betaHPV mediated skin carcinogenesis.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.06.019.

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Own substantial contributions to this publication:

- Performed ELISAs and analyzed the data shown in Fig. 1;
- Performed whole protein as well as phospho-proteomics analyses shown in Fig. 2 and Fig. 4;
- Carried out phospho-kinase assays and Prime-PCR (RT-qPCR), analyzed and shown in Fig. 3
- Interpreted all data
- Wrote the manuscript and revised the paper in response to reviewer comments.

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Novel Insights Into Cellular Changes in HPV8-E7 Positive Keratinocytes: A Transcriptomic and Proteomic Analysis

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Human papillomavirus type 8 (HPV8) is associated with the development of non-melanoma skin cancer. In the past we already delved into the mechanisms involved in keratinocyte invasion, showing that the viral E7 oncoprotein is a key player that drives invasion of basal keratinocytes controlled by the extracellular protein fibronectin. To unravel further downstream effects in E7 expressing keratinocytes we now aimed at characterizing gene and protein/phosphoprotein alterations to narrow down on key cellular targets of HPV8-E7. We now show that gene expression of GADD34 and GDF15 are strongly activated in the presence of E7 in primary human keratinocytes. Further analyses of fibronectin-associated factors led to the identification of the Src kinase family members Fyn and Lyn being aberrantly activated in the presence of HPV8-E7. Phospho-proteomics further revealed that E7 not only targets cell polarity and cytoskeletal organization, but also deregulates the phosphorylation status of nuclear proteins involved in DNA damage repair and replication. Many of these differentially phosphorylated proteins turned out to be targets of Fyn and Lyn. Taken together, by using unbiased experimental approaches we have now arrived at a deeper understanding on how fibronectin may affect the signaling cascades in HPV8 positive keratinocytes, which may be key for skin tumorigenesis and that may also aid in the development of novel therapeutic approaches for betaHPV-mediated cancers.

Keywords: betapapillomavirus, skin cancer, E7 oncoprotein, keratinocyte invasion, fibronectin

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INTRODUCTION

Cutaneous squamous cell carcinoma (SCC) is the most common metastatic skin cancer, and its incidence is increasing worldwide. DNA damage, as a consequence of excessive UV light exposure, is known to be the main causative factor for the development of cutaneous SCC which arises from a precancerous lesion termed actinic keratosis (Fania et al., 2021). Recent epidemiological

Abbreviations: ECM, extracellular matrix; PHAK, primary human adult keratinocyte; PHFK, primary human foreskin keratinocyte; DED, de-epidermalized human dermis.

data strongly implicate that human papillomavirus (HPV) of genus betapapillomavirus (betaHPV) has a co-factorial role in this process (Hasche et al., 2017, 2018; Hufbauer and Akgül, 2017). The oncogenic potential of betaHPV in skin carcinogenesis was originally identified in patients suffering from the rare inherited disease Epidermodysplasia verruciformis (EV), who have an increased susceptibility to betaHPV infections (Howley and Pfister, 2015). However, betaHPV can also be found in skin cancers of non-EV patients. Particularly, immunosuppressed patients, such as organ-transplant-recipients, also have a higher susceptibility to betaHPV infections of the skin as well as an increased risk of developing SCC compared with healthy individuals (Bouwes Bavinck et al., 2018; Rollison et al., 2019; Smola, 2020).

Despite the more detailed analyses of the viral E6 oncoprotein in keratinocyte transformation, the precise molecular mechanisms by which the E7 oncoprotein may interact with the host cells are still being investigated. We have previously described already that the E7 protein of the oncogenic betaHPV type 8 (HPV8) plays a pivotal role in inducing keratinocyte hyperproliferation and invasion when expressed in primary human adult keratinocytes (PHAK) differentiating in de-epidermalized human dermis (DED)-based organotypic skin cultures (Akgül et al., 2005; Heuser et al., 2016). These keratinocytes display hyperproliferation in the regenerated epithelium and are positive for both cyclin E and p16INK4a, indicating that E7 is able to overcome p16INK4a induced cell cycle arrest (Westphal et al., 2009). In addition, HPV8-E7 positive keratinocytes lose their physiological polarity and invade the underlying dermis (Akgül et al., 2005). Here, downward migration of keratinocytes is facilitated by the degradation of basement membrane components such as collagen VII, collagen IV and laminin V, a process that is paralleled by increased expression of the matrix metalloproteinases (MMP)-1, MMP-8, and membrane-type (MT)1-MMP (Akgül et al., 2005; Smola-Hess et al., 2005). In light of the fact that E7 positive keratinocytes do not exhibit invasive behavior on a collagen type I matrix (Boxman et al., 2001), but do so when cultivated as DED-based organotypic skin cultures, we hypothesized that components of the extracellular matrix (ECM) may be critical in eliciting the invasive behavior of HPV8-E7 positive cells. To address the mechanism by which E7 mediates invasion we tested for a possible cadherin-switch which is commonly seen in invasion-related epithelial-mesenchymal transition. To this end, we examined the E-cadherin and N-cadherin mRNA expression in cells cultured on collagen type IV, laminin V, or fibronectin. Only when HPV8-E7 positive keratinocytes were grown on fibronectin, a reduction in E-cadherin and an increase in N-cadherin levels were detected. These data identified fibronectin as an inducer of the observed cadherin-switch in the presence of HPV8-E7. In subsequent experiments we could detect increased fibronectin levels in peritumoral areas in betaHPV positive SCC as opposed to betaHPV negative tissues. In addition, we showed that E7 positive keratinocytes have elevated levels of integrin α3β1 on their cell surface. Silencing of the α3 chain and the usage of the invasion deficient E7 mutant L23A led to a drastic reduction in the invasive potential of HPV8-E7 cells, thus providing evidence for a direct role of the fibronectin/α3β1 integrin axis in invasion of HPV8-E7 expressing keratinocytes (Heuser et al., 2016). The overall goal of the present follow-up project therefore was to identify further gene expression and protein/phospho-protein alterations using unbiased global transcriptomic and proteomic approaches in order to further pinpoint key downstream cellular targets of HPV8-E7 which may extend our knowledge on the mechanisms underlying betaHPV associated skin carcinogenesis.

MATERIALS AND METHODS

Cell Culture

N/TERT keratinocytes (Dickson et al., 2000) and the spontaneously immortalized human skin keratinocyte cell line PM1 (Proby et al., 2000) were cultivated either in KGM-Gold (0.05 mM Calcium, Lonza, Cologne, Germany) or in RM+ medium (consisting of a 3:1 ratio of Dulbecco's modified Eagle's medium (DMEM) and DMEM-F12, supplemented with 10% fetal calf serum (FCS), 1% glutamine, 0.4 μg hydrocortisone, 10^{-10} M cholera toxin, 5 μg/mL transferrin, 2×10^{-11} M liothyronine, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, 1 × penicillin–streptomycin mixture) (Hufbauer et al., 2013). The PT67 cell line was maintained in DMEM with 10% FCS and penicillin-streptomycin mixture. All cell lines were cultivated at 37°C and 6% CO_2.

Retroviral Expression Vectors and Infection of Keratinocytes

The generation of the pLXSN-based retroviral vectors coding for HPV8 E1, E2, E6, E7, or E6E7 has been previously described (Akgül et al., 2005, 2007; Leverrier et al., 2007). Production of retroviruses and retroviral transduction of N/TERT and PM1 keratinocytes were performed as previously described (Hufbauer et al., 2013). Briefly, keratinocytes were seeded out in 6 cm dishes. Next day, retroviral supernatants were mixed with an equal volume of DMEM in the presence of 5 μg/mL of hexadimethrine bromide (polybrene) and added to the keratinocytes. Spin infection was made by centrifugation for 1 h at 300 × g. Cells were then washed with PBS and cultured further for 2 days. Then, cells were selected against G418 (500 μg/mL) until only infected keratinocytes had survived. In subsequent experiments we used pooled stable cell populations to minimize possible variations due to the randomness of the viral integration site in the cellular chromosomes.

RTqPCR and PrimePCR

To quantify mRNA levels of cellular genes, quantitative reverse transcription-PCR (RTqPCR) using the LightCycler system (Roche, Mannheim, Germany) was performed as previously described (Hufbauer et al., 2015). The primers used for this study were:

ATF3-fw: TTTGCCATCCAGAACAAGC;
ATF3-rev: CATCTTCTTCAGGGGCTACCT;
GADD34-fw: GCTTCTGGCAGACCGAAC;

GADD34-rev: GTAGCCTGATGGGGTGCTT;
GDF15-fw: CCCGGGACCCTCAGAGTT;
GDF15-rev: CCGCAGCCTGGTTAGCA;
HPRT1-fw: TGACACTGGCAAAACAATGCA;
HPRT1-rev: GGTCCTTTTCACCAGCAAGCT.

The RTqPCR based PrimePCR (Fibronectin-Binding Integrin in Cell Motility H96, BIORAD, Feldkirchen, Germany) was performed in quadruplicate using 96-well plates with pre-spotted primers in each well of the plate. The plates were designed to be analyzed using a Roche LC 480 light cycler, using the SsoAdvanced^{TM} Universal SYBR® Green Supermix. Analyses of these plates were carried out following manufacturer's instructions.

DNA Microarrays

The cDNA microarray analyses performed in the Akgül group used total RNA extracted from monolayer cultures of PHAKs in total passage 3 (passage 2 after transduction). The RNA was analyzed in biological triplicates from cells containing either the empty vector control pLXSN or pLXSN-HPV8-E7. Changes in gene expression were measured using the Human Genome U133A arrays according to Affymetrix protocols. Microarray data were analyzed using Transcriptome Analysis Console software (Thermo Fisher Scientific, v4.0.1.36). For analysis of differential gene expression, we applied the following conditions to determine statistically relevant genes: (a) ANOVA test: ebayes, (b) a fold change cut-off of < -2 or > 2, and (c) determination of statistical significance with $p < 0.05$. Gene ontology analysis was carried out using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) database (v14.1). These microarray data files have been uploaded to the Gene Expression Omnibus (GEO) database (GSE133813) (Kirschberg et al., 2019).

The cDNA microarray analyses performed in the Alonso group at the DKFZ were performed with RNA isolated from primary keratinocytes also transduced with pLXSN or pLXSN-HPV8-E7 coding retroviruses. The Affymetrix chip-based experiments and data analyses were performed at the core facility of the German Cancer Center (DKFZ) (**Supplementary Table 1**). Differentially expressed genes (DEG) from both independent studies were overlapped to minimize bias of the data in respect to genetic variations among keratinocyte donors, cell culture media effects, thus making the results more robust.

ELISA

The Human GDF15 Quantikine ELISA Kit (RnD systems) was used to measure GDF15 in cell culture supernatants. For this N/TERT-8E7 were cultured on fibronectin and cell culture supernatants were harvested after 4 days. Collected supernatants were treated with protease/phosphatase inhibitors (Protease/Phosphatase Inhibitor Cocktail, Cell Signaling). The ELISA was performed according to the manufacturer's protocol.

Western Blotting and Phospho-Kinase Array Analysis

Western blotting was performed as previously described (Marx et al., 2018). Briefly, cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors, incubated on ice for 30 min, sonicated and then centrifuged at 15,000 × g at 4°C for 15 min. Protein concentrations were measured with the Pierce^{TM} BCA Protein Assay Kit (Thermo Fisher Scientific, Dreieich, Germany). SDS gels were transferred upon completion to nitrocellulose membranes, which were then blocked with either 5% bovine serum albumin or 5% dry milk in TBST [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20]. The primary antibodies used targeted 14-3-3β (sc-59419, clone 60C10, Santa Cruz), ERK1/2 (#4695, clone 137F5, Cell Signaling), Fyn (#4023, Cell Signaling), p-Tyr^{530}-Fyn (#PA5-36644, Invitrogen), Lyn (#4576, clone 5G2, Cell Signaling), p-Tyr^{397}-Lyn (#orb6340, Biobyrt, Cambridge, United Kingdom), GAPDH-Alexa680 (sc-365062, cloneG9, Santa Cruz). HuT78 whole cell lysates (sc-2208, Santa Cruz) were used as positive controls. Antibodies were incubated for 2 h at RT or overnight at 4°C. Following washing, membranes were incubated with corresponding secondary antibodies (IRDye, LI-COR, Lincoln, Nebraska, United States). Western blots were visualized by employing the LI-COR ODYSSEY FC Imaging system with the Image Studio Ver 5.2. software.

The Human Phospho-Kinase Array Kit (RnD Systems, Minneapolis, United States), spotted with antibodies targeting phospho-sites in kinases associated with malignant processes, was used to identify potentially deregulated kinases in HPV8-E7 expressing keratinocytes grown for 96 h on fibronectin coated tissue culture plates. Cells were rinsed with PBS and then lysed using the provided buffer supplemented with 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, and 10 μg/mL Pepstatin. The lysates were gently incubated at 4°C for 30 min and then centrifuged at 14,000×g for 5 min, before transferring the supernatants into a clean test tube. Samples were then further processed according to the manufacturer's protocol. Visualization of the membranes and evaluation of individual spot densities were carried out employing the LI-COR ODYSSEY FC Imaging system with the Image Studio Ver 5.2. software.

Proteomics

Proteomic and phospho-proteomic analyses were performed with total cell extracts from HPV8-E7 expressing N/TERT keratinocytes (N/TERT-8E7) grown for 96 h on fibronectin coated tissue culture plates which were lysed using 8M urea supplemented with protease and phosphatase inhibitors. The whole cell lysates were prepared in triplicates in two separate experiments ($n = 6$) were analyzed at the proteomics core facility of the Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD) by means of "stable isotope labeling with amino acids in cell culture" (SILAC)-based mass-spectrometry. The protein profile was submitted to the Proteomics Identifications Database (PRIDE) and is available via ProteomeXchange with the identifier PXD026099. Briefly, samples were reduced with DTT (100 mM) and alkylated with 550 mM Iodoacetic acid (IAA) prior to digestion with trypsin and Lys-C overnight at 37°C. Generated peptides were extracted by incubation with increasing amount of acetonitrile, concentrated in a

speed-vac and primed prior to LC-MS/MS analysis by the STAGE tip technique (Rappsilber et al., 2003). For liquid chromatography and tandem mass spectrometry (LC-MS/MS), an easy nLC 1,000 (Thermo Fisher Scientific) was coupled to the quadrupole-based Q Exactive Plus (Thermo Fisher Scientific) instrument by a nano-spray ionization source. Peptides were separated on a 50 cm in-house-packed column by a two-solvent buffer system: buffer A (0.1% formic acid) and B (0.1% formic acid in acetonitrile). The content of buffer B was increased from 7 to 23% within 40 min and followed by an increase to 45% in 5 min and a washing and re-equilibration step before continuing with the injection of the next sample. Proteins were considered differentially expressed or phosphorylated under the following criteria: (a) $\log_2$fold change < -2.5 or > 2.5, (b) p-value of $p < 0.05$, and (c) same phosphorylation status in all data sets. Data analysis was performed using the Perseus software suite, KinasePhos2.0 and ShinyGO (v0.61). The differentially phosphorylated protein targets of E7 were imported into the STRING database (Szklarczyk et al., 2019) to identify relevant protein-protein interactions and functional enrichment networks. STRING holds physical as well as functional interaction information derived from high-throughput experimental data, published literature, and computationally predicted co-expression studies.

For secretomic analyses the supernatants of N/TERT-8E7^{wt}, N/TERT-8E7^{L23A} as well as empty vector control grown for 96 h on fibronectin coated tissue culture plates ($n = 6$) were harvested and treated with protease/phosphatase inhibitors (Protease/Phosphatase Inhibitor Cocktail, Cell Signaling). Afterward, the supernatants were concentrated using Amicon ultra-15 centrifugation 3K tubes (Merck Millipore, Darmstadt, Germany). Concentrated supernatants as well as a media-only control were then further processed at the CECAD proteomics facility and subjected to mass-spectrometric analysis. The profile of secreted proteins was submitted to the PRIDE Database and is available via ProteomeXchange with the identifier PXD026100.

Statistical Analysis

Microarray data analyses were analyzed using ANOVA test, setting a $\log_2$fold change < -2.5 or > 2.5 with a p-value of $p < 0.01$. Gene ontology analysis was carried out using PANTHER (v14.1) and ShinyGO (v0.61). RTqPCRs were repeated at least three times in duplicate, and the results are presented as mean ± standard deviation (SD). Statistical significance was determined with an unpaired 2-tailed Student's t-test calculated with GraphPad Prism (Version 9.0). Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control conditions ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Images from immunoblots are from a representative experiment and were qualitatively similar in $n = 3$ experiments. The ANOVA two sample t-test was performed to identify statistically significant ($p < 0.05$) peptides in proteomics and the phospho-proteomic data sets.

RESULTS

Identification of HPV8-E7 Gene Targets

We previously published a cDNA oligonucleotide microarray (Affymetrix) data set based on HPV8-E7 expressing PHAK which had been grown in KGM-Gold (Kirschberg et al., 2019). In parallel to our own works similar experiments had been carried out by the Alonso group at the DKFZ in Heidelberg using primary human foreskin keratinocytes (PHFK) (**Supplementary Table 1**). Both experiments were carried out with cells grown on uncoated cell culture dishes. In an effort to identify similarities in gene expression profiles in these HPV8-E7 positive keratinocytes we created an overlap of both sets. After filtering and normalization ($\log_2$fold-change < -2.5 or > 2.5 and $p < 0.01$) we identified a total of 81 and 73 genes, respectively, in the Cologne and Heidelberg data sets. Interestingly, using these highly stringent cut-off values we still found an overlap of 4 genes (**Figure 1A**). Three of these genes, namely ATF3 (activating transcription factor 3), GADD34 (growth arrest and DNA damage-inducible protein), and GDF15 (growth differentiation factor–15) were found to be upregulated in both data sets and may therefore be the most relevant target genes of HPV8-E7. In contrast, the THBS1 (Thrombospondin 1) gene showed an opposite regulation which is why this gene was occluded from further analysis (**Figure 1B**).

In order to further validate our results in cutaneous keratinocytes, the PM1 cell line (Proby et al., 2000) was used which is one of very few keratinocyte lines that can be cultivated in different cell culture growth media. This enabled us to grow keratinocytes under both undifferentiating (KGM-Gold, low-calcium) as well as differentiating (RM+, high-calcium) conditions using the same cell line. Furthermore, in order to underpin the importance of the interaction of E7 with cellular genes the PM1 cells were not only retrovirally transduced with vectors harboring the E7 gene, but also in parallel with constructs expressing E1, E2, or E6.

In the PM1 cell line our experiments showed that E1 and E2 do not have an influence on GDF15 expression. We could, however, confirm a significant effect of HPV8-E7 on GDF15 under non-differentiating conditions, unveiling a fivefold upregulation in gene expression. Most intriguingly, we observed a cumulative effect of E6 and E7 on GDF15 with an up to 15-fold increase in gene expression. Curiously E6 expression alone had appeared to have a slight suppressive effect on GDF15 expression (**Figure 1C**). Somewhat unexpectedly, these effects on gene expression were greatly diminished, yet still significant under differentiating growth conditions (**Figure 1D**). Since GDF15 is a member of the transforming growth factor beta superfamily (TGFβ) and therefore a secretory protein we next tested via ELISA whether the elevated GDF15 gene expression may also result in higher protein contents in cell culture supernatants. As shown in **Figure 1E**, an increase in GDF15 protein levels could also be confirmed in cell culture supernatants of cells grown under non-differentiating conditions. Despite the fact that an upregulation of ATF3 had been predicted in the microarray analyses we could not confirm this by means of RTqPCR.

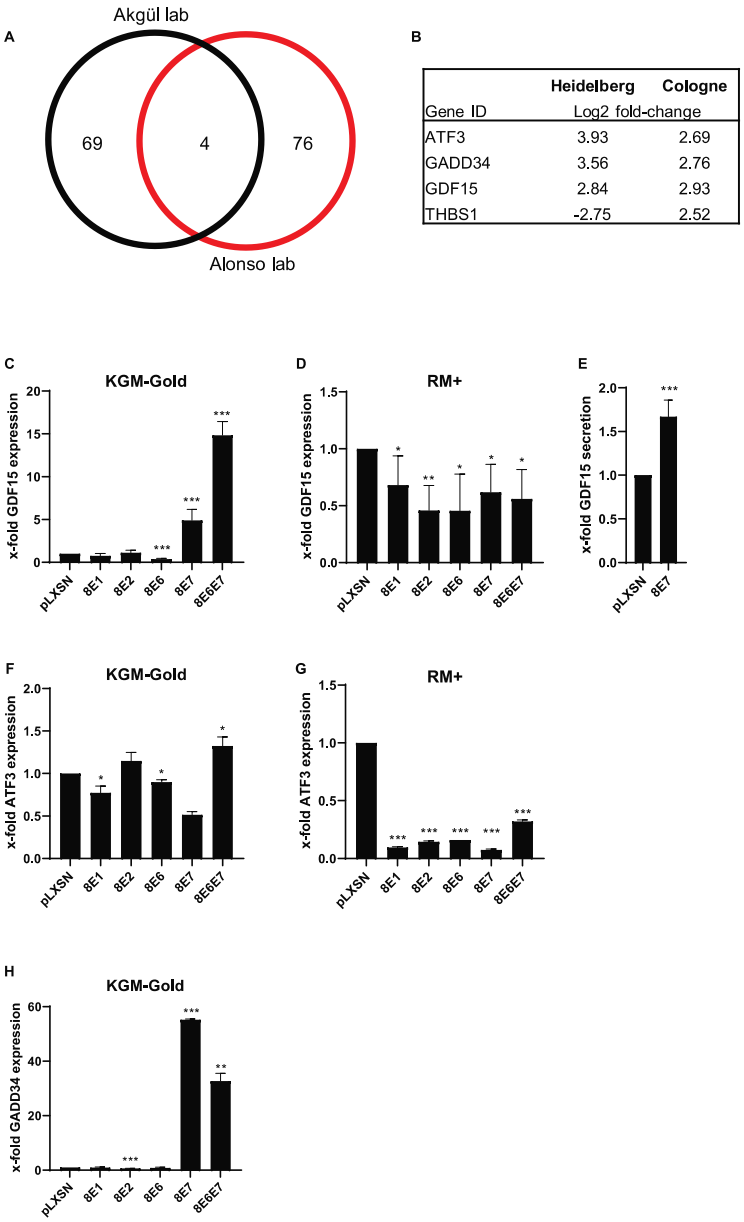


FIGURE 1 | Transcriptional changes in HPV8-E7 positive keratinocytes. **(A)** Venn Diagram comparison of two independent cDNA microarray analyses carried out with PHAK expressing HPV8-E7 in the Akgül lab in Cologne and with PHFK expressing HPV8-E7 in the Alonso lab at the DKFZ in Heidelberg. **(B)** Gene IDs of 4 genes differentially expressed in both array studies. **(C,D)** GDF15 mRNA expression in isogenic PM1 keratinocytes cultured either in KGM-Gold or RM+, respectively. **(E)** GDF15 ELISA performed with cell culture supernatants of HPV8-E7 expressing keratinocytes grown in KGM-Gold medium. **(F,G)** ATF3 mRNA expression in isogenic PM1 keratinocytes cultured either in KGM-Gold or RM+, respectively. **(H)** GADD34 mRNA expression in PM1 keratinocytes cultured in KGM-Gold. RTqPCRs were normalized to HPRT1 mRNA levels ($n = 3$ independent experiments performed in duplicate). Error bars represent standard deviations. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control conditions (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

All viral proteins seem to only exert neglible control over ATF3 under undifferentiating conditions (**Figure 1F**). Under differentiating conditions, however, all virus genes have a strong and highly statistically significant suppressive effect on ATF3 gene expression (**Figure 1G**). The most profound effect of E7 on gene expression was measured for GADD34 and to a lesser degree for E6E7 positive cells under undifferentiating conditions. The presence of E1, E2, and E6 did not have any effect (**Figure 1H**). GADD34 gene expression could not be detected at all in isogenic keratinocytes grown in RM+ medium, implying that GADD34 may only play a vital role in undifferentiated basal keratinocytes (data not shown).

HPV8-E7 Mediated Alterations in Fibronectin Associated Signaling Pathways

Since we already knew that the ECM protein fibronectin is key in the regulation of cell fate and also has an important effect on invasive behavior of HPV8-E7 positive keratinocytes (Heuser et al., 2016), we first investigated how HPV8-E7 may alter the secretome of keratinocytes relevant for a crosstalk between epidermal keratinocytes and the ECM. Here, we cultivated both the N/TERT-8E7wt, N/TERT-8E7^{L23A} (which codes for the invasion deficient mutant) as well as the empty vector control on a fibronectin matrix and collected the cell culture supernatants after 96 h. In the mass-spectrometric analysis we unveiled 11 proteins that were exclusively differentially secreted in N/TERT-8E7 cells compared to control and N/TERT-8E7^{L23A} (**Figures 2A,B** and **Supplementary Table 2**). A further GO enrichment analysis pointed toward most of the identified proteins (except for GALNT2 and UGP2) to be involved in processes associated with cell growth and proliferation (**Figure 2C**). Interestingly, 14-3-3 family members, which are known to be scaffolds that facilitate a multitude of protein-protein interactions, were present in these pathways and may therefore represent an important regulatory axis. In order to check if the reduced quantity of 14-3-3 in the secretome was the result of decreased protein expression we next performed Western blots with cell extracts of the same cells. Here, we could again show that the presence of HPV8-E7 had a suppressive effect on 14-3-3 β expression (**Figure 2D**).

To narrow down on gene expression changes of known fibronectin-associated genes in HPV8-E7 positive keratinocytes we next performed PrimePCRs using the Fibronectin-Binding Integrin in Cell Motility H96 assay. These RTqPCRs were performed in quadruplicate, with RNA derived from both control as well as HPV8-E7 positive N/TERT grown on fibronectin coated cell culture dishes for 96 h. The PrimePCR revealed four genes that were more than 15-fold up- or down regulated, namely PIK3R2 (phosphoinositide-3-kinase regulatory subunit 2), Fyn as well as RhoA (ras homolog family member A) and Cfl1 (cofilin 1), respectively (**Figure 3A**). Since Src kinase family members, like Fyn, are known to be key modulators of cancer cell invasion and metastasis including the skin (Serrels et al., 2009), we next performed Western blots and could confirm higher protein levels and observed four major splice variants. Interestingly, Fyn phosphorylation at pTyr530, which is a negative regulatory tyrosine residue for this kinase (Qayyum et al., 2012) was weaker in E7 cells compared to the empty vector control (**Figure 3B**).

This observation sparked the idea to have a deeper look at differentially phosphorylated kinases in HPV8-E7 positive keratinocytes. We therefore used a phosphokinase array kit (Proteome Profiler^{TM} Array) with total cell extracts from N/TERT-8E7 grown on fibronectin-coated tissue culture plates for 96 h. Based on the obtained experimental data we discovered that now particularly the Src kinase family member Lyn seemed to be strongly hyperphosphorylated in HPV8-E7 positive cells (**Figure 3C**). Subsequent Western blot analyses proved that Lyn was indeed hyperphosphorylated at residue Tyr397 in HPV8-E7 positive keratinocytes whilst total protein levels remained unchanged (**Figure 3D**). Taken together, our findings show that Src-kinases are deregulated in HPV8-E7 positive keratinocytes grown on fibronectin.

Identification of Differentially Phosphorylated Proteins in HPV8-E7 Positive Cells

Considering that protein kinases appear to play an important role in downstream signaling from fibronectin in HPV8-E7 positive cells we next expanded our studies to investigate the phospho-proteome of these cells. We first performed total proteome and phospho-proteome analyses of HPV8-E7 positive cells compared to control cells using the Perseus software suite. After subtracting the total proteins from the phopho-proteins we report 40 statistically significant phospho-proteins in HPV8-E7 expressing keratinocytes. Out of these 40 proteins, 16 were hyperphosphorylated and 24 proteins hypophosphorylated (**Figure 4**). The Perseus output table, showing the kinase target motif enrichments, excluding the results for Fyn and Lyn kinases, additionally predicts 14-3-3 binding motifs for several hypo- and hyperphosphorylated proteins as presented in **Supplementary Table 3**.

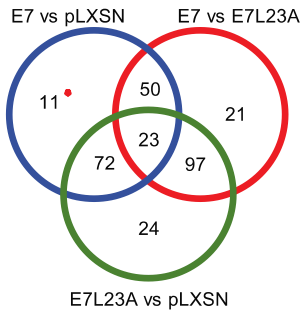
To pinpoint potential Fyn and Lyn targets, we performed an additional kinase motif enrichment analysis using the online tool KinasePhos2.0 (Wong et al., 2007). This analysis revealed that a great number of the identified differentially phosphorylated proteins are targets of the Src kinase family members Fyn and Lyn which may point toward HPV8-E7 hijacking these kinases to enact post-translational modifications in HPV8 infected cells (**Figures 4A,C**).

These aberrantly phosphorylated proteins could be grouped into two enriched pathway clusters, namely "cytoskeletal organization and cell polarity" and "DNA replication and repair" (**Figures 4B,D**). STRING database analyses further revealed that a majority of the identified proteins form protein-protein interaction networks (**Supplementary Figure 1**).

DISCUSSION

HPV8-E7 is the main viral oncoprotein exerting control over both proliferation and invasive behavior of keratinocytes

A



B

Gene ID	Description	fold-change in secretion levels
PITPNB	Phosphatidylinositol transfer protein beta isoform	8.8
GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	7.2
IGFBP7	Insulin-like growth factor-binding protein 7	6.7
UGP2	UTP--glucose-1-phosphate uridylyltransferase	5.7
PRDX6	Peroxiredoxin-6	4.5
SFN	14-3-3 protein sigma	-4.8
YWHAB	14-3-3 protein beta/alpha	-4.9
LGMN	Legumain	-4.9
CTSC	Cathepsin C	-5.5
PRDX1	Peroxiredoxin-1	-5.6
CNPY2	Protein canopy homolog 2	-7.9

C

N	GO category	Genes
6	Response to stress	CTSC IGFBP7 LGMN PRDX1 PRDX6 SFN
5	Catabolic process	CTSC LGMN PRDX1 PRDX6 YWHAB
5	Regulation of signaling in response to stimulus	CTSC LGMN PRDX1 SFN YWHAB
5	Regulation of biological quality	LGMN PRDX1 PRDX6 SFN YWHAB
4	Cell proliferation	IGFBP7 LGMN PRDX1 SFN
4	Regulation of multicellular organismal process	CNPY2 CTSC LGMN SFN
3	Regulation of localization and molecular function	LGMN SFN YWHAB
3	Macromolecule localization	PITPNB SFN YWHAB
3	Regulation of growth	LGMN IGFBP7 SFN
3	Regulation of developmental process	CTSC LGMN SFN

D

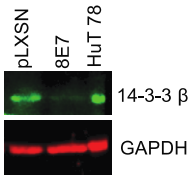


FIGURE 2 | Secretome analysis of N/TERT-8E7^{wt} or N/TERT-8E7^{L23A} grown on fibronectin. **(A)** Venn diagram depicting a three set comparison of differentially secreted proteins taken from N/TERT-pLXSN, N/TERT8-E7^{wt}, or N/TERT8-E7^{L23A}. The starred number indicates proteins targeted only by HPV8-E7^{wt}. **(B)** Description of differentially proteins exclusively secreted by HPV8-E7^{wt} positive keratinocytes as depicted in subfigure A The log2-fold changes of the secretome results from N/TERT-8E7 and N/TERT-pLXSN were compared to calculate the difference. **(C)** Gene ontology analysis of differentially secreted proteins using ShinyGO v0.61. **(D)** Representative immunoblots of RIPA extracts from N/TERTs expressing HPV8-E7 tested for 14-3-3β protein expression (n = 3). HuT78 whole cell lysate served as a positive control. Equal loading was confirmed by immunoblotting for GAPDH.

(Akgül et al., 2005, 2007; Heuser et al., 2016; Hufbauer and Akgül, 2017). More detailed molecular insights on the mechanisms involved would therefore be invaluable to further expand our understanding of how these invasive processes are initiated and propagated. In order to shed more light on these issues this comprehensive study employed several different unbiased experimental angles, including transcriptomic, secretomic and proteomic/phospho-proteomic approaches.

From previous studies we already knew that a broad range of genes deregulated in HPV8-E7 positive primary keratinocytes are controlled upstream through Sp1/Sp3 binding sites in the promoter region (Kirschberg et al., 2019). Here, we now compared our data with a data set generated in the Alonso laboratory, with both data sets having been generated with either PHAK or PHFK, both grown on plastic. We thus identified three genes, namely ATF3, GADD34, and GDF15, as they showed the

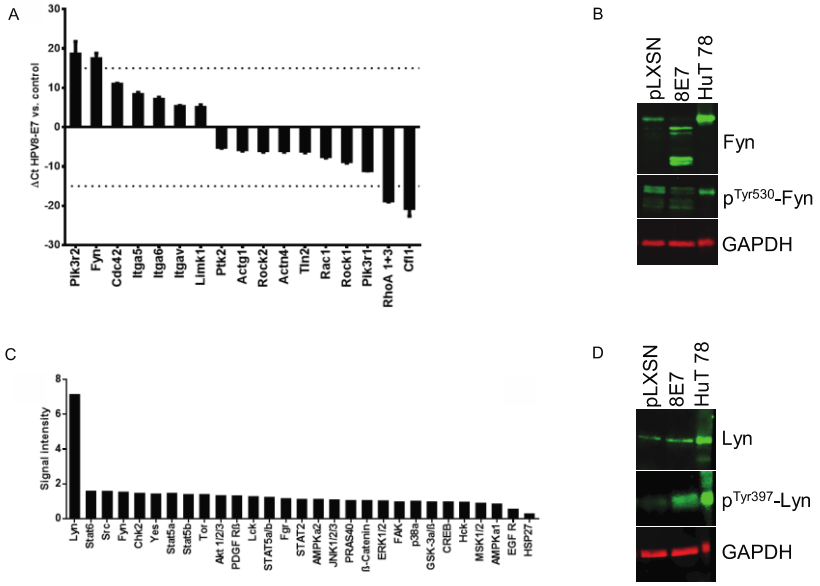


FIGURE 3 | Analysis of fibronectin associated gene expression and protein kinase phosphorylation changes in HPV8-E7 positive keratinocytes. **(A)** PrimePCR results for N/TERT-8E7 grown on fibronectin for 96 h (n = 4). **(B)** Representative immunoblots of RIPA extracts from N/TERT-8E7 tested for total Fyn and p^{Tyr530}-Fyn (n = 3). HuT78 whole cell lysates served as a positive control. Equal loading was confirmed by immunoblotting for GAPDH. **(C)** Protein kinase phosphorylation patterns in N/TERT-8E7 cells cultured on fibronectin for 96 h were analyzed using the Proteome Profiler Human Phospho- Kinase Array Kit (RnD Systems). The cut-off value for up- and down-regulated genes was set to fivefold. The dashed line indicates the 15-fold change. **(D)** Representative immunoblots of RIPA extracts of N/TERT-8E7 tested for total Lyn and p^{Tyr397}-Lyn (n = 3). HuT78 whole cell lysates served as a positive control. Equal loading was confirmed by immunoblotting for GAPDH.

same expression pattern in the cDNA data sets of both studies. By means of RTqPCR we could validate both GADD34 and GDF15 as novel gene targets of E7 but not ATF3. One possible reason for this discrepancy could be that ATF3 might only be specifically targeted in primary keratinocytes but not in a cell line. Interestingly, the upregulation of GADD34 and GDF15 could only be detected in keratinocytes grown under undifferentiating conditions (grown in KGM-Gold), thus confirming these genes as novel gene targets of HPV8-E7. Despite the fact that both genes are known to be regulated by Sp1/Sp3 the exact mechanism by which this upregulation is achieved in HPV8-E7 positive cells should be further researched in future studies.

E7 co-expression with E6 even had a synergistic effect on GDF15 over-expression under undifferentiating conditions. In contrast, co-expression had a repressive effect on GADD34, whose expression was, however, still highly elevated compared to the control. When isogenic keratinocytes were grown under differentiating conditions the activating effect of E7 on GDF15 expression was abolished. Under these conditions, the GADD34 transcripts could not be detected at all pointing toward an exclusive role in undifferentiated keratinocytes. GDF15 is a member of the TGF-β superfamily. However, its role in keratinocyte biology has thus far not been extensively studied. It is known that GDF15 overexpression in melanoma cells is associated with tumor invasion and metastasis (Unal et al., 2015). In line with our data, it is reasonable to speculate that the E7 mediated upregulation and secretion of GDF15 may disturb the physiological keratinocyte differentiation program. This may also have an influence on epidermal-dermal crosstalk, which may ultimately result in aberrant cell growth. GADD34, on the other hand, belongs to a family of genes which are induced by DNA damage and apoptotic cell death, and which are also known to enhance the apoptotic response to DNA damage (Grishin et al., 2001). In later studies, GADD34 was shown to dephosphorylate several kinases that function in various important signaling cascades (Collier et al., 2017). Our observation that GADD34 is only detectable and thus seemingly targeted by E7 in undifferentiated keratinocytes with a basal cell phenotype points toward a role of this regulation axis in manipulating the growth of undifferentiated basal keratinocytes.

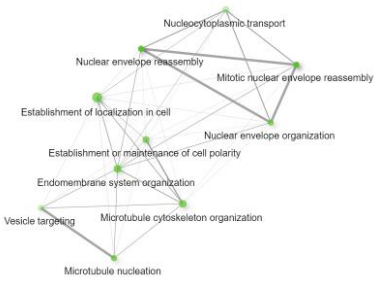
Since previous results of our group had shown the importance of fibronectin in modulating the phenotype of HPV8-E7 expressing keratinocytes we now aimed at identifying signaling molecules and cascades that are altered under these conditions.

Our secretomic and proteomic data both hint at a possible involvement of 14-3-3 family members in HPV8-E7 mediated

A

ID	log2fc	phosphorylation	Fyn/Lyn motif enrichment
SRRM2	4.00	Hyperphos.	Lyn
CEP170B	3.72	Hyperphos.	-
CEP170	3.06	Hyperphos.	-
SPAG9	2.40	Hyperphos.	-
RBMX	2.37	Hyperphos.	Fyn
DOCK7	2.35	Hyperphos.	Fyn
CSNK1D	2.35	Hyperphos.	-
EMD	2.34	Hyperphos.	Fyn, Lyn
TANC2	2.32	Hyperphos.	Fyn, Lyn
RANBP2	2.30	Hyperphos.	-
NFIA	2.26	Hyperphos.	Fyn
TMPO	2.20	Hyperphos.	-
CLASP2	2.16	Hyperphos.	-
LMNA	2.11	Hyperphos.	-
ZNF687	2.06	Hyperphos.	Fyn, Lyn
CDC42EP1	2.02	Hyperphos.	-

B



C

ID	log2fc	phosphorylation	Fyn/Lyn motif enrichment
NDRG1	-2.00	Hypophos.	Fyn, Lyn
MYO1E	-2.04	Hypophos.	Fyn, Lyn
AHNAK	-2.05	Hypophos.	Fyn, Lyn
BRIP1	-2.07	Hypophos.	-
SIPA1L1	-2.08	Hypophos.	Fyn
MAGI3	-2.12	Hypophos.	-
PLEKHG3	-2.17	Hypophos.	Lyn
ZNF281	-2.22	Hypophos.	-
MCM4	-2.24	Hypophos.	-
U2SURP	-2.24	Hypophos.	-
PPIP5K2	-2.25	Hypophos.	-
PSIP1	-2.28	Hypophos.	Fyn, Lyn
PHRF1	-2.35	Hypophos.	Fyn, Lyn
PTRF	-2.38	Hypophos.	-
LEMD3	-2.40	Hypophos.	-
INCENP	-2.40	Hypophos.	Fyn
PRRC2C	-2.47	Hypophos.	-
RAP1GAP2	-2.56	Hypophos.	-
MDC1	-2.72	Hypophos.	Fyn, Lyn
ANLN	-2.74	Hypophos.	-
SF3B1	-2.82	Hypophos.	Fyn, Lyn
EPN3	-3.09	Hypophos.	-
COL17A1	-3.20	Hypophos.	Lyn
RPS6	-4.42	Hypophos.	-

D

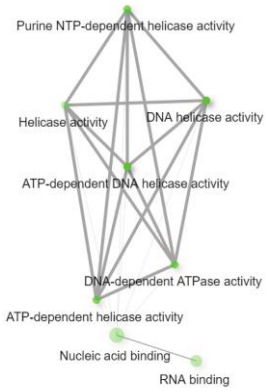


FIGURE 4 | Phospho-proteome analysis of N/TERT-8E7 cultured on fibronectin. **(A)** List of proteins found to be hyper-phosphorylated in between HPV8-E7 positive keratinocytes. **(B)** Gene Ontology Enrichment Analysis of hyper-phosphorylated proteins. **(C)** List of proteins found to be hypo-phosphorylated in between HPV8-E7 positive keratinocytes. **(D)** Gene Ontology Enrichment Analysis of hypo-phosphorylated proteins. Fyn and Lyn kinase substrates identified using KinasePhos2.0 were highlighted in **(A,C)**.

cellular changes. The 14-3-3 family is comprised of a number of scaffolding proteins that serve as platforms for several protein-protein interactions. The 14-3-3 protein family fulfills various regulatory functions involved in signaling pathways, cell proliferation, DNA damage response, apoptosis, differentiation and cell survival. By altering key regulatory proteins 14-3-3 family

members play pivotal roles in cancer initiation and progression (Khorrami et al., 2017; Liu et al., 2021). It is therefore not unreasonable to assume that the observed E7-mediated changes in 14-3-3 protein levels as well as changes in global protein phosphorylation patterns may also lead to changes in 14-3-3 binding motif availabilities as well. Considering that we found a downregulation of 14-3-3 protein levels both in cell culture supernatants as well as in full cell lysates our experimental results may thus have unveiled a modulatory axis hijacked by the HPV8-E7 oncoprotein which may contribute to disturbing physiological keratinocyte functions.

Using different experimental approaches and bioinformatical tools we identified the Src kinase family members Fyn and Lyn, among others (**Figures 3,4** and **Supplementary Table 3**), as novel crucial kinases deregulated by E7 that may play a vital role in altering the phosphorylation profile of cellular proteins involved in cytoskeletal organization and cell polarity as well as proteins associated with DNA replication and repair, which may ultimately contribute HPV8-E7 mediated keratinocyte transformation.

In that light, Src kinases are comprised of a family of 11 non-receptor tyrosine kinases and interestingly only Fyn and Lyn seem to become over-abundantly activated in HPV8-E7 positive keratinocytes grown on fibronectin. Here, Lyn expression has also been implicated to be activated in response to DNA damage. Also, Lyn—but not other Src kinases—is activated following genotoxic stress, where Lyn is associated with cell cycle arrest and apoptotic processes (Yoshida et al., 2000). Furthermore, Lyn is known to act as a negative regulator of GADD34 in DNA damage-induced cell death (Grishin et al., 2001). As in HPV8-E7 positive cells Lyn is overly activated one would expect now that GADD34 expression would also be repressed (as described above). Surprisingly, this does not seem to be the case, which points toward an uncoupling of this regulatory circuit in the presence of E7 and E6E7, respectively. Moving on to Fyn, this kinase has three known splice variants, namely FynT, FynB, and FynC, which arise from alternative splicing of exon 7 of the Fyn gene (Brignatz et al., 2009; Uddin et al., 2020). The observation of a fourth Fyn signal in Western blots of HPV8-E7 positive cells grown on fibronectin may potentially have unveiled a fourth unknown Fyn splice variant, which may be associated with E7 expression and contribute to the complexity of Src kinase regulation in betaHPV positive skin. Interestingly, in a previous study on HPV16-E7 the authors also described phosphorylation of Src family kinases Yes and Fyn in the presence of HPV16-E7 (Szalmas et al., 2013).

In addition to the already known HPV8-E7 binding partners (Enzenauer et al., 1998; Rozenblatt-Rosen et al., 2012; Sperling et al., 2012; White et al., 2012; White and Howley, 2013; Grace and Munger, 2017; Oswald et al., 2017, 2019) we now identified novel cellular targets which are differentially regulated in HPV8-E7 positive keratinocytes. The finding that the Src kinases Fyn and Lyn are significantly over-activated and likely change the phosphorylation status of several proteins when cells are grown on fibronectin may possibly represent the most profound results of our study. As Src kinase inhibitors already exist our results may therefore pave the way for novel therapeutic approaches in which these inhibitors could be used for treatment of skin SCCs in which betaHPV involvement has been demonstrated.

DATA AVAILABILITY STATEMENT

The original microarray data presented in the study have been deposited in the Gene Expression Omnibus (GEO) repository, accession number (GSE133813). The proteomics data for differentially phosphorylated proteins as well as for the secreted proteins are available at the ProteomeXchange repository, accession numbers (PXD026099) and (PXD026100), respectively.

AUTHOR CONTRIBUTIONS

MK, ASS, HGD, SH, AW-H, AA, and MH performed the experiments. MK, ASS, HGD, SH, AW-H, AA, MH, and BA analyzed the data. MK and BA designed the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.672201/full#supplementary-material

Supplementary Figure 1 | STRING protein-protein interaction network. Interaction network of hyper-phosphorylated and hypo-phosphorylated protein targets of HPV8-E7. All nodes indicate the proteins and lines indicate the interaction between two proteins.

Supplementary Table 1 | CDNA microarray data analysis from the Alonso group, DKFZ, Heidelberg.

Supplementary Table 2 | Precompiled list of the secretome analysis.

Supplementary Table 3 | Precompiled list of the phospho-proteome analysis.

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3.3 Virus Genes. 2019; 55:600–609

BetaHPV E6 and E7 colocalize with NuMa in dividing keratinocytes. Oswald E*, **Kirschberg M***, Aubin F, Alonso A, Hufbauer M, Akgül B, Auvinen E.

*Equal contribution.

Own substantial contributions to this publication:

- Created organotypic skin cultures expressing HPV8-E7;
- Performed immunofluorescence stainings against NuMa on organotypic skin cultures, human skin cancers as well as HPV8 transgenic mouse skin shown in Fig. 2;
- Re-worked all figures.
- Wrote the manuscript and revised the paper in response to reviewer comments.

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ORIGINAL PAPER

BetaHPV E6 and E7 colocalize with NuMa in dividing keratinocytes

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Abstract
Human papillomaviruses (HPVs) of genus betapapillomavirus (betaHPV) are implicated in skin carcinogenesis, but their exact role in keratinocyte transformation is poorly understood. We show an interaction of HPV5 and HPV8 oncoproteins E6 and E7 with the nuclear mitotic apparatus protein 1 (NuMA). Binding of E6 or E7 to NuMA induces little aneuploidy, cell cycle alterations, or aberrant centrosomes. Intracellular localization of NuMA is not altered by E6 and E7 expression in 2D cultures. However, the localization profile is predominantly cytoplasmic in 3D organotypic skin models. Both viral proteins colocalize with NuMA in interphase cells, while only E7 colocalizes with NuMA in mitotic cells. Intriguingly, a small subset of cells shows E7 at only one spindle pole, whereas NuMA is present at both poles. This dissimilar distribution of E7 at the spindle poles may alter cell differentiation, which may in turn be relevant for betaHPV-induced skin carcinogenesis.

Keywords Human papillomavirus · Betapapillomavirus · E6E7 oncoproteins · Nuclear mitotic apparatus protein 1

Introduction

A role for human papillomaviruses (HPVs) of genus betapapillomavirus (betaHPV) in the development of nonmelanoma skin cancer among immunosuppressed and immunocompetent individuals has been proposed for decades. The oncogenic potential of betaHPV in skin carcinogenesis was originally identified in patients suffering from the rare inherited disease epidermodysplasia verruciformis (EV), who have an increased susceptibility to betaHPV infections. However, betaHPV can also be found in skin cancers of non-EV patients (reviewed in [1–3]). Recently, a prospective epidemiological study provided compelling evidence that betaHPV diversity and viral loads in the skin are associated with squamous cell carcinogenesis [4]. Further support for an etiologic role arose from the observation that betaHPV is frequently detected in actinic keratoses, which are premalignant lesions of the skin [5]. Curiously, despite frequent detection of betaHPV DNA in premalignant lesions of immunocompetent patients, viral sequences are rarely found in skin tumors in these patients, pointing towards a hit-and-run mechanism by betaHPV in the carcinogenic process [6, 7].

To prove the oncogenic potential of betaHPV early proteins in vivo, HPV8 transgenic mice were generated, in which the expression of the complete early genome region (CER) is under the control of the human keratin-14 promoter (K14-HPV8-CER). These animals develop papillomas, partially along with moderate to severe dysplasia and squamous cell carcinoma (SCC) [8, 9].

Mechanistically, HPV5 and HPV8 E7 positive cells display hyperproliferation and are positive for both cyclin E and p16INK4a, indicating that E7 is able to overcome p16INK4a-induced cell cycle arrest [10]. BetaHPV E7 oncoproteins bind pRb with lower affinity than high-risk

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alphaHPV [11], and this binding is accompanied by degradation of pRb [12]. In particular, the E7 oncoprotein of HPV8 proved to be the major mediator of keratinocyte invasion in 3D organotypic skin cultures. E7 positive cells lose their polarity and migrate downwards into the extracellular matrix (ECM), concomitant with upregulation of matrix metalloproteases (MMP) [13]. In addition, E7-mediated invasion is directed by overexpression of integrin α3β1 and interaction of E7 positive cells with fibronectin in the ECM [14].

It has previously been shown that HPV6, HPV11, and HPV16 E7 proteins bind to nuclear mitotic apparatus protein 1 (NuMA) leading to the introduction of mitotic errors and aneuploidy [15]. NuMA is indispensable in organizing microtubules at centrosomes and in chromosome alignment during mitosis [16–18]. Furthermore, post-mitotic nuclear reassembly is dependent on NuMA, and NuMA is also a crucial structural component of the nuclear matrix during interphase (reviewed in [19]). Another key function of NuMA is the control of cell polarity, which is important for asymmetric division of basal epithelial cells [20, 21].

In search for cellular interaction partners of HPV5 and HPV8 E6 and E7, we identified NuMA as a binding partner for these viral proteins and describe relevance of this association for keratinocyte homeostasis.

Materials and methods

Cell culture

293T and COS-7 cells were cultured in DMEM supplemented with 10% FCS, penicillin, and streptomycin. COS-7 cells were used in immunofluorescence experiments because they are easy to transfect. Primary human keratinocytes (PHKs) were cultured in KGM-2 Medium (Promocell, Heidelberg, Germany). Phoenix cells were cultured as previously described [22] for the generation of recombinant retroviruses.

Plasmids and transfections

Retroviral constructs for the expression of HPV5 and HPV8 E6, E7, E6E7 and HPV16 E6E7 were generated using the pLXSN vector. These constructs were transfected into Phoenix cells, and retroviruses were subsequently isolated and used to transduce PHK cells as previously described [22]. HPV5 E7 mutants I2P, Δ26-29, E31G, R66E, and Δ79-83 cloned into pcDNA3.1(−) were obtained from Dr. Ramon Garcia-Escudero (CIEMAT, Madrid, Spain) and then further subcloned into a pGEX4T3 vector for the expression of N-terminal GST fusion proteins. Expression constructs pCMV-SPORT-3xFLAG-E6 or -E7 of HPV5 or HPV8 were generated for expression of fusion proteins coding for triple flag-tagged viral proteins [23] to be used in immunoprecipitation experiments. HPV5 and HPV8 E6 and E7 genes were subcloned into pCMV to express fusion proteins with the AU1 epitope tag for immunofluorescence analyses.

Yeast two-hybrid system

In order to identify cellular proteins binding to HPV5 E6 or E7 oncogenes, the Matchmaker two-hybrid system was used together with a cDNA library from the HaCaT human keratinocyte cell line (Clontech, Saint-Germain-en-Laye, France). Either E6 or E7 was used as a bait. Identification of binding proteins was performed by MALDI-TOF as previously described [24].

GST pulldown experiments

Preparation of GST fusion proteins and GST pulldown experiments were performed as previously described [23]. In these experiments both wild-type (wt) HPV5 and HPV8 E7 as well as the HPV5 E7 mutants I2P, Δ26-29, E31G, R66E, I67R, and Δ79-83 were used.

Immunoblotting, immunoprecipitation, and confocal microscopy

Primary antibodies used in immunoblotting, immunoprecipitation, and immunofluorescence are described in Supplementary Table 1. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with the corresponding antibodies, and detection was carried out using ECL. Immunoprecipitation of flag-tagged E6 or E7 proteins was performed as previously described [23]. For immunofluorescence experiments, cells were grown on coverslips and subsequently transfected with expression constructs, fixed with 3% paraformaldehyde, washed, and incubated with primary antibodies. Secondary antibodies were labeled with Alexa488 or Alexa594 (Invitrogen, Carlsbad, CA). Images were generated using a Zeiss LSM 700 or a Leica TCS SP microscope.

Cell cycle and DNA content analysis

Transduced and control PHK cells were treated for 24 h with nocodazol, fixed, stained with propidium iodide, and analyzed for DNA content by FACS. The experiments were repeated twice, each time with two biological replicates. FACS analyses were performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using fixed cells. The data were analyzed using CellQuest (BD Biosciences) as well as FlowJo (Tree Star, Ashland, OR) as described elsewhere [25]. Transduced as well as control PHK cells were exposed to BrdU for 45 min, fixed and stained using fluorescein-labeled BrdU

antibodies (Supplementary Table 1). BrdU incorporation was measured using FACS analysis.

Centrosome number quantification

In order to quantify centrosome numbers, PHK were transduced with retroviruses, fixed in PFA and then stained with antibodies targeting pericentrin. DNA was visualized by DAPI staining. The experiment was repeated twice, and the centrosome numbers were calculated in a minimum of 200 cells per experiment with comparable results.

UV irradiation of the K14-HPV8-CER and FVB/n wt mice

Mouse lines used in this study included FVB/n wt (Charles River Laboratories, Sulzfeld, Germany) and the transgenic hemizygous FVB/N line K14-HPV8-CER [8]. UV irradiation protocols were approved by the governmental animal care office North-Rhine-Westphalia (Leibnizstraße 10, 45659 Recklinghausen, Protocol No. 8.87-50.10.35.08.163) and were in accordance with the German Animal Welfare Act as well as the German Regulation for the protection of animals used for experimental purposes. For UV treatment, age (5 weeks) and sex-matched mice were shaved and irradiated once with 10 J/cm^2 UVA and 1 J/cm^2 UVB on a 4 cm^2 sized dorsal caudal area. All offspring were macroscopically examined for the presence of skin lesions on day 24 after UV treatment. On day 24 after UV irradiation the animals were sacrificed and samples of the irradiated skin were collected, fixed, and subsequently embedded in paraffin.

Sections of EV skin lesions and organotypic cultures

Human EV biopsy material from archival paraffin blocks taken during routine surgical excision (for detailed information see [26]) was used in this study. Ethical approval for the use of human samples was obtained from the Ethics Committee of the Medical University of Warsaw.

The generation of organotypic skin cultures of keratinocytes expressing HPV5, HPV8, or HPV16 E7 was based on a de-epidermalized human dermis serving as the dermal equivalent, which was then repopulated with PHKs. These 3D cultures were grown for 14 days at the air–liquid interphase, followed by fixing and embedding in paraffin [10].

Results

Identification and validation of NuMA among binding partners of betaHPV E6 and E7

In order to identify putative interaction partners of E6 and E7 of HPV5, yeast two-hybrid screens were performed. Among the putative E6 interaction partners we identified NuMA. Subsequent in vitro GST pulldown experiments confirmed the interaction between NuMA and E6 as well as E7 of HPV5 and HPV8 (Fig. 1a). Furthermore, 293T cells were transfected separately with pCMV-SPORT-3xFLAG-E6 or -E7 expression constructs coding for flag-tagged HPV5 and HPV8 E6 or E7 proteins. Immunoprecipitation with extracts from transfected cells was then performed with anti-flag antibodies. Again, NuMA coprecipitated with E6 and E7 proteins of both HPV5 and HPV8, and E6 binding was consistently shown to be stronger than E7 binding (Fig. 1b).

NuMA binding is independent of the pRb binding site of E7

Binding of HPV16 E7 to NuMA is known to be dependent on the pRb binding domain of E7 [15]. In order to analyze whether betaHPV might share this property we performed binding experiments using N-terminal GST fusion constructs of wt HPV5 E7 as well as the E7 variant Δ26-29, known to be defective in respect to pRb binding [27] and additional constructs mutated at positions I2P (abrogating hydrophobicity), E31G, R66E, I67R (which all changed the charged at their respective positions) or Δ79-83 (deletion of the zinc finger domain [28]). The GST pulldown experiments showed no significant reduction in NuMA binding by any of the tested E7 mutants (Fig. 1c). We therefore concluded that regions outside the pRb binding and zinc finger domain are required for NuMA binding by HPV5 E7.

Expression of NuMA in betaHPV-positive skin

In order to characterize NuMA expression in betaHPV-positive differentiating epithelia, we first studied the staining pattern of NuMA in the skin of K14-HPV8-CER transgenic mice. These mice develop skin tumors 3 weeks following UV exposure, whereas the FVB/n wt mice heal from UV-induced hyperplasia [9, 29]. In FVB/n wt skin, which healed from UV-induced hyperplasia, NuMA is mainly expressed in the hair follicles, with weak staining of the interfollicular epidermis (Fig. 2a). In skin tumors of K14-HPV8-CER mice, elevated expression of NuMA throughout the epithelium was observed. In healthy human skin, nuclear NuMA appeared to be evenly distributed throughout the

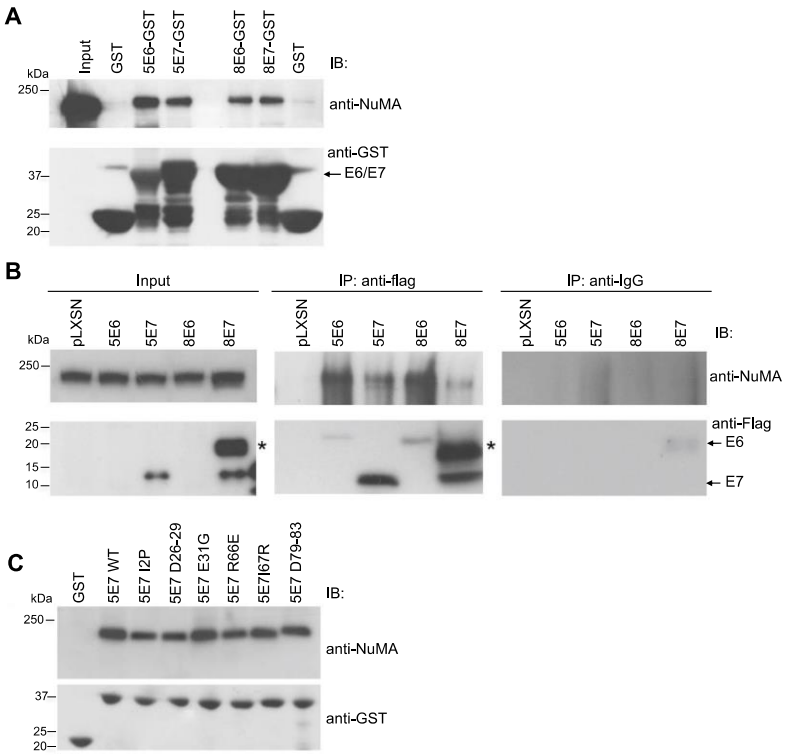

Fig. 1 HPV5 and HPV8 E6 and E7 bind to NuMA. **a** Lysates of 293T cells were subjected to GST pulldown using HPV5 and HPV8 E6 or E7 GST fusion proteins. One-tenth of the extract was used as loading control. Antibodies against GST and NuMA were used in immunoblotting. **b** 293T cells were transfected with expression constructs coding for flag-tagged HPV5 and HPV8 E6 and E7 proteins. Cell lysates were analyzed by immunoprecipitation and immunoblotting using antibodies against the flag epitope or NuMA *E7 multimer. **c** NuMA binding by wild-type and mutant HPV5 E7 proteins. 293T cell lysates were subjected to GST pulldown using wild-type or mutant GST E7 fusion proteins. Immunoblotting was performed with antibodies against GST or NuMA

basal cell layer with decreasing staining intensity toward the skin surface. In skin SCC sections from EV patients we detected different staining patterns. For instance, in section EV-a, NuMA is mainly nuclear but also cytoplasmic and restricted to the suprabasal cell layers. Unexpectedly, in the skin SCC section EV-b NuMA has a mainly cytoplasmic staining profile (Fig. 2b). Interestingly, in organotypic skin cultures of control keratinocytes, NuMA is exclusively cytoplasmic. The same holds true for the HPV8 E7 skin culture with stronger staining intensity in the suprabasal cell layers (Fig. 2c).

E6 and E7 and NuMA colocalize in interphase

We next studied the subcellular localization of E6 or E7 and NuMA by means of immunofluorescence and laser confocal microscopy. Cells were transfected with expression constructs coding for AU1-tagged E6 or E7 fusion proteins of HPV5 and HPV8. The cells were then fixed and stained with a specific antibody targeting AU1. Nuclear NuMA expression in interphase COS-7 cells was shown, which proved not to be altered by expression of HPV5 or HPV8, E6 and E7 (Fig. 3a). Similar findings were obtained in transfected 293T cells (data not shown). The E7 proteins colocalized with NuMA in interphase nuclei. Manders coefficient for colocalization was 0.45–0.54 for E6 and 0.78–0.91 for E7 (Supplementary Fig. 1). Additional weak cytoplasmic staining was shown for the E6 protein. Next, we looked at a possible colocalization of AU1-tagged HPV5 E7 mutants with NuMA. All mutants colocalized with NuMA in the nucleus, comparable to wt E7. Manders coefficient for colocalization was 0.8–0.85 for the different mutants (Supplementary Fig. 1). This result is in line with the GST pulldown experiments, further reinforcing the notion that interaction with NuMA is not dependent on the pRb binding domain of HPV5 E7.

E7 but not E6 colocalizes with NuMA at mitotic spindle poles

A central function of NuMA is the alignment and stabilization of microtubules of the mitotic spindle. We therefore studied the localization of E6 and E7 of both HPV5 and HPV8 as well as NuMA in cells undergoing mitosis. AU1-tagged E6 and E7 proteins were expressed in COS-7 cells, and the cells were fixed and further analyzed using

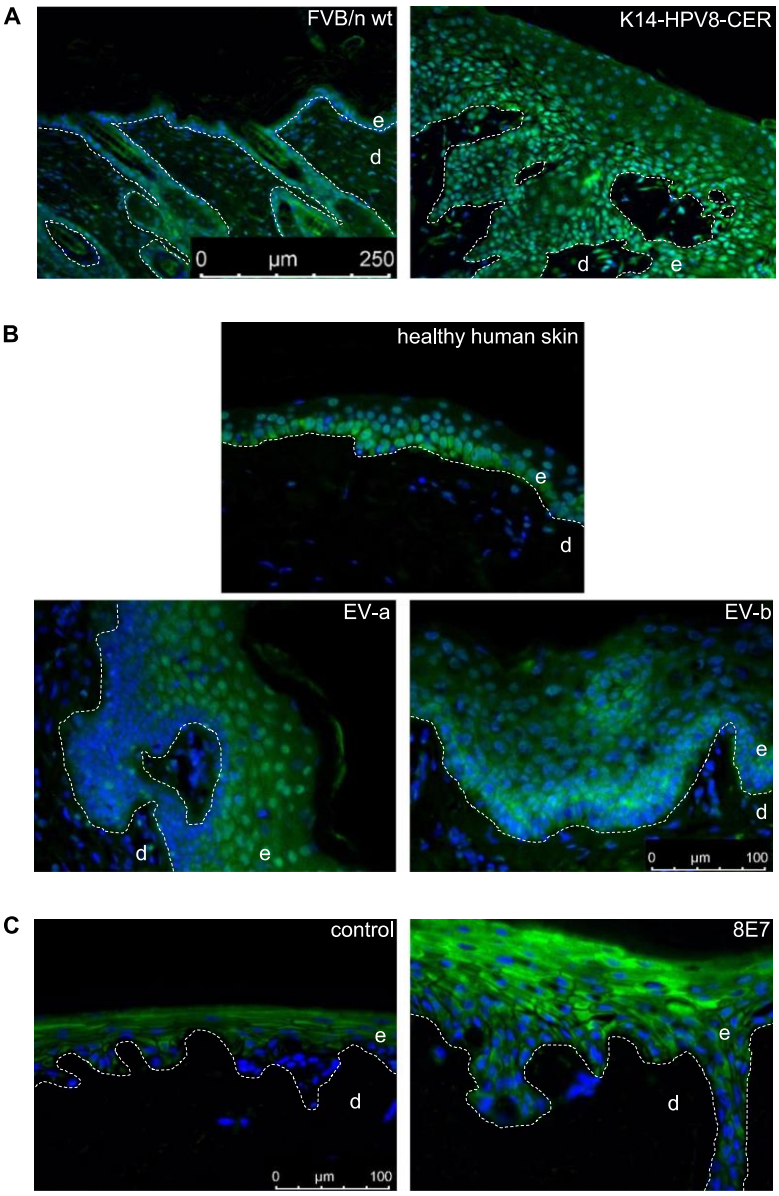

Fig. 2 NuMA expression in HPV8 positive skin. **a** Sections of UV-irradiated skin from FVB/n wt (top left) and K14-HPV8-CER mice (top right), harvested 24 days after UV treatment, were stained for NuMA. **b** Representative immunofluorescence staining of NuMA in healthy skin and two individual skin SCC from EV patients positive for HPV5, 8, 20, 23, 36, 50. **c** Representative immunofluorescence staining of NuMA in organotypic skin cultures, which were repopulated with PHK harboring the empty retroviral vector pLXSN (control) or HPV8 E7, respectively, and grown for 14 days at the air–liquid interphase (*blue* DAPI, *green* NuMA, *dashed line* basement-membrane zone, *d* dermis, *e* epidermis)

immunofluorescence and laser confocal microscopy. Both HPV5 and HPV8 E7 were found to colocalize with NuMA at spindle poles in mitotic cells (Fig. 3b). Surprisingly, no colocalization of E6 with NuMA was observed during mitosis. Most intriguingly, in a small subset of cells, E7 of both HPV5 and HPV8 was seen at only one spindle pole in dividing cells, whereas NuMA still localized at both poles (Fig. 3b).

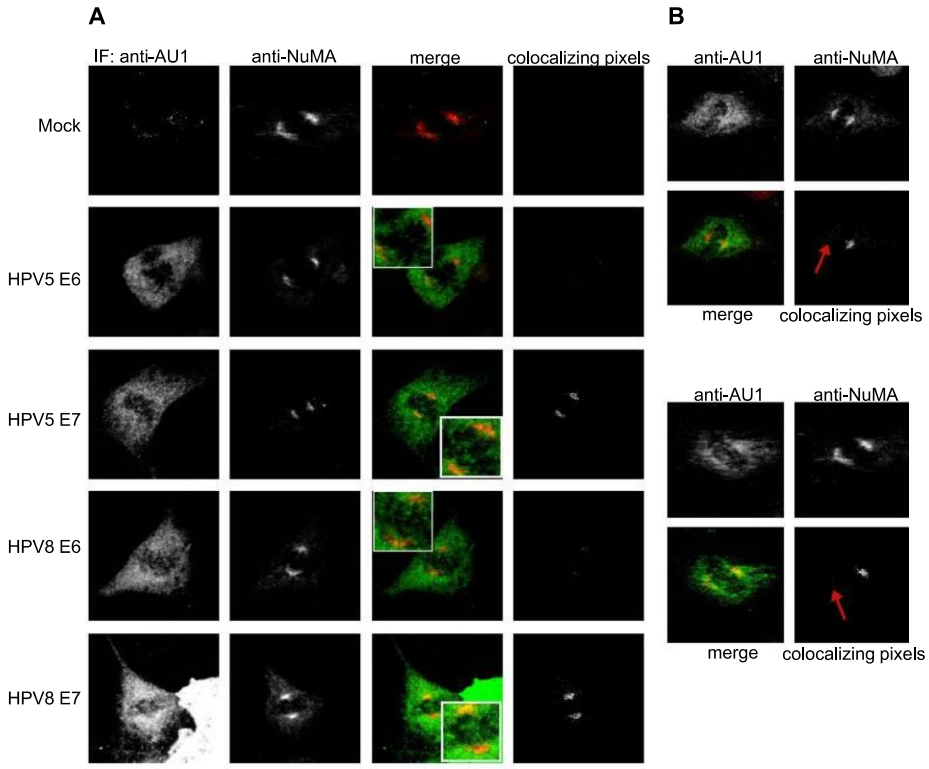

Fig. 3 E6 and E7 and NuMA colocalize in interphase. **a** COS-7 cells were transfected with expression plasmids for HPV5 or HPV8 E6 or E7 provided with AU1 epitope. Fixed cells were stained with antibodies to AU1 (green) or NuMA (red). The images show *z*-axis projections of 15–18 layers. Colocalized pixels were calculated using the Colocalization Highlighter Plugins of ImageJ. Only those pixels which exceeded the cutoff intensity of 100 (8 bit) and whose intensities on both channels showed at least 95% correlation were taken into account. **b** E7 and NuMA colocalize at mitotic spindle poles. Immunofluorescence showing NuMA (red) and AU1-E7 (green) in COS-7 cells expressing either HPV5 or HPV8 E7. The two separate four-field images show stainings in two individual cells (red arrow: indicates spindle pole without E7)

HPV5 or HPV8 E7 do not induce marked aneuploidy

Based on our observation that E7 and NuMA colocalize in mitotic cells we next asked whether this might interfere with NuMA function during mitosis. In particular, we were interested to see whether the possible interference may impact the stability or lead to misalignment of chromosomes at the mitotic spindle in metaphase. As such associations have previously been described for HPV16 [30–33], we transduced PHK cells with HPV5, HPV8 or with HPV16 E6E7 retroviral constructs for comparison. After undergoing selection, the cells were stained with propidium iodide and analyzed by FACS. No increase in DNA content above 4N was observed for any of the different viral proteins (Supplementary Fig. 2a, c). We next treated the transduced PHK cells with nocodazole, a reagent inhibiting polymerization of microtubuli and causing cell cycle arrest through activation of the spindle checkpoint. The increase in the > 4N cell population still remained insignificant, except for HPV16 E6E7, which showed a 5.5-fold increase in the > 4N population as compared to empty vector control (Supplementary Fig. 2b, c). Collectively, we conclude that betaHPV E6E7 proteins do not markedly induce aneuploidy in epithelial cells in vitro.

Cell cycle is not altered due to betaHPV E7

Interaction of HPV16 E7 with NuMA leads to destabilization of the mitotic complex of NuMA with dynein and consequently to chromosomal misalignment in prometaphase. This may in turn result in a prolonged mitosis, as has already been shown for HPV16 in NIH3T3 cells [15]. We therefore

wanted to explore whether the interaction of betaHPV oncogenes with NuMA may affect the cell cycle in epithelial cells in a similar fashion. To this end, nonsynchronized PHK cells were transduced with pLXSN constructs coding for the different E6E7 proteins. The cells were then incubated with bromouridine, fixed, stained, and examined by FACS analysis. None of the E6E7 proteins disrupted the cell cycle profile to a significant degree (Supplementary Fig. 3a, b). Prolonged prometaphase should have led to an increased cell population in the G2/M phase which was, however, not observed for any of the E6E7 constructs (Supplementary Fig. 3a, b). The experiment was repeated in 293T and NIKS cells with comparable results (data not shown). The > 4N cell population was only found to be increased in HPV16 E6E7 expressing cells (Supplementary Fig. 3a, b), which was in line with the results from the nocodazole experiment described above (Supplementary Fig. 2b, c).

BetaHPV E7 does not induce aberrant centrosome distribution

PHK were transduced with retroviral constructs for E6E7 proteins, fixed, and stained for pericentrin, and the number of centrosomes was counted. HPV5 or HPV8 E6E7 caused minimal changes in centrosome numbers, whereas the changes due to HPV16 E6E7 expression were considerable. We conclude that while HPV16 E6E7 have a strong effect on centrosome duplication, neither HPV5 nor HPV8 E6E7 appears to have this ability (Fig. 4).

Discussion

The role of alphaHPV in cancers at mucosal sites such as the anogenital region and the head and neck region is well established [34]. How betaHPV is involved in skin carcinogenesis is not well understood as betaHPV DNA is less frequently found and is only present in negligible copies per cell in skin cancers. In this respect, a hit-and-run mechanism has been proposed for betaHPV in skin carcinogenesis, a hypothesis which is further supported by experimental models [35–37], suggesting that betaHPV acts as a tumorigenic trigger but is dispensable at later tumor stages. Both alphaHPV and betaHPV exert certain tumorigenic functions. For instance, they both bind pRb [11], which leads to its degradation [12]. Furthermore, it has been shown that HPV16 E7 binds to NuMA, and the binding motif is located within the pRb binding domain [15]. We now show that betaHPV E7 proteins are also capable of binding NuMA. However, the interacting region does not seem to be located within the pRb binding domain, contrary to HPV16 E7. In interphase cells, NuMA is a key component of the nuclear matrix, and it is also involved in the reassembly of the nucleus following mitosis (reviewed in [38]). During mitosis, NuMA orchestrates correct organization of microtubules at centrosomes and proper chromosome alignment in preparation of cell division. Contrary to HPV16 E7 [39, 40], only a small proportion of betaHPV E6E7 expressing cells display extra centrosomes, suggesting that there is only a minor interference with centrosome separation upon betaHPV E6E7 binding to

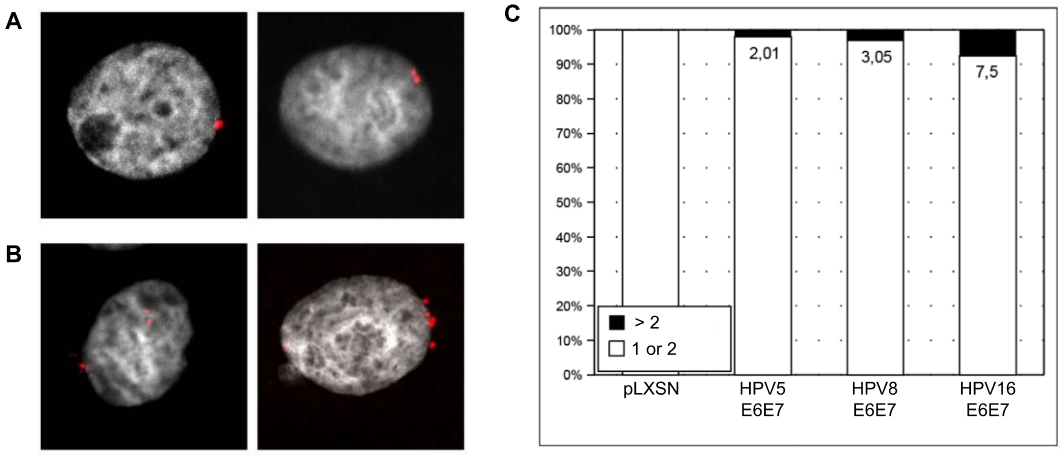

Fig. 4 PHK were transduced with pLXSN vector, or constructs expressing E6 and E7 of either HPV5, HPV8 or HPV16, and the cells were stained for pericentrin (red). **a** Left image: control; right image: HPV5-E6E7. **b** Left image: HPV8-E6E7; right image: HPV16-E6E7. **c** Proportions in percentages of HPV5, HPV8 or HPV16 E6E7 expressing cells with more than two centrosomes. The experiment was repeated twice, and each time 100–200 cells were taken into account

NuMA. In summary, we report that betaHPV E6E7 binding to NuMA has only a minor effect in respect to cellular DNA content, cell cycle or the number of centrosomes, which has also been shown in the past for HPV16 E7 [41].

Although we did not observe relocalization of NuMA due to betaHPV oncogene binding in 2D tissue cultures, ECM signaling-dependent redistribution of NuMA has previously been shown in carcinogenesis and in differentiation [42]. We therefore analyzed NuMA distribution patterns in the skin of K14-HPV8-CER transgenic mice, in HPV8 positive EV skin SCC, as well as in 3D organotypic skin cultures of HPV8 E7 expressing keratinocytes. In FVB/n wt murine skin as well as skin tumors from transgenic mice, NuMA is located in the nucleus. Unexpectedly, the analyses of SCC skin from different EV patients showed distinct NuMA staining patterns. We observed nuclear localization but, intriguingly, we also found tumors with mainly cytoplasmic NuMA. Curiously, in human 3D skin cultures, which can also be regarded as a wound healing model, NuMA was exclusively cytoplasmic. HPV8 E7 did not affect the distribution, but rather the total expression levels in suprabasal cells. Whether the two distinct NuMA staining patterns are linked to distinct cells of origin of skin SCC [43], or whether wound healing processes may affect the subcellular distribution of NuMA are questions which would most certainly warrant further investigation.

In addition to symmetric cell division, NuMA has an important function in establishing cell polarity required for asymmetric cell division taking place in, e.g., basal epithelial cells, where one daughter cell retains its basal identity, whereas the other cell is committed to differentiation. NuMA is responsible, together with dynein, for positioning the minus ends of microtubules at the poles of the mitotic spindle [44]. A dysfunction of NuMA–dynein complexes may therefore lead to failure in spindle formation and incorrect orientation of metaphase chromosomes. We now show that HPV8 E6E7 proteins bind and colocalize with NuMA in interphase nuclei. However, only E7 associates with NuMA at the poles of the mitotic spindle. It is intriguing that in a small fraction of cells E7 colocalizes with NuMA at only one of the spindle poles. It is tempting to speculate that the asymmetric E7 distribution might alter the proportions of basal or stem cells undergoing asymmetric versus symmetric division in infected skin. Asymmetric basal cell division is a prerequisite for epithelial differentiation, and it enables and supports the completion of the HPV replication cycle. A potential shift of the balance toward symmetric cell division in the basal cell layer would, however, increase the number of stem-like cells, and possibly trigger neoplastic development. We recently provided further support for this idea by showing that particularly the expression of HPV8 E7 in vitro increases the number of stem-like cells in colony-formation and tumor sphere assays, thus facilitating the initiation of oncogenic events, which is also relevant for the hit-and-run hypothesis of betaHPV-mediated skin carcinogenesis [45]. Understanding the molecular basis of NuMA distribution may be relevant for asymmetric/symmetric division in betaHPV-infected epithelia and should be a topic for further research.

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Author contributions EO, MK, FA, and MH performed the experiments. AA and EA conceived and designed the study. AA, BA, and EA analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethical approval for the use of skin biopsies of epidermodysplasia verruciformis patients for scientific purposes was obtained from the Ethics Committee of the Medical University of Warsaw.

Informed consent All authors have reviewed the final version of the manuscript and approve it for publication.

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ATP synthase modulation leads to an increase of spare respiratory capacity in HPV-associated cancers. **Kirschberg M**, Heuser S, Marcuzzi GP, Hufbauer M, Seeger JM, Đukić A, Tomaić V, Majewski S, Wagner S, Wittekindt C, Würdemann N, Klussmann JP, Quaas A, Kashkar H, Akgül A.

Own substantial contributions to this publication:

- Performed immunofluorescence stainings against ATP5B and supervised immunohistochemical stainings against ATP5B performed by Prof. Dr. Quaas at the Institute of pathology, Cologne, shown in Fig. 1E and Fig 1F, respectively;
- Generated retrovirally transduced keratinocytes coding for HPV E7 and used these cells in Seahorse experiments shown in Fig. 3;
- Interpreted the results and performed statistical analysis of OPSCC stainings for ATP5B together with Dr. Würdemann shown in Fig. 4;
- Wrote the manuscript and revised the paper in response to reviewer comments.

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ATP synthase modulation leads to an increase of spare respiratory capacity in HPV associated cancers

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Mucosal and skin cancers are associated with infections by human papillomaviruses (HPV). The manner how viral oncoproteins hijack the host cell metabolism to meet their own energy demands and how this may contribute to tumorigenesis is poorly understood. We now show that the HPV oncoprotein E7 of HPV8, HPV11 and HPV16 directly interact with the beta subunit of the mitochondrial ATP-synthase (ATP5B), which may therefore represent a conserved feature across different HPV genera. By measuring both glycolytic and mitochondrial activity we observed that the association of E7 with ATP5B was accompanied by reduction of glycolytic activity. Interestingly, there was a drastic increase in spare mitochondrial respiratory capacity in HPV8-E7 and an even more profound increase in HPV16-E7 expressing cells. In addition, we could show that ATP5B levels were unchanged in betaHPV positive skin cancers. However, comparing HPV-positive and HPV-negative oropharyngeal squamous cell carcinomas (OPSCC) we noticed that, while ATP5B expression levels did not correlate with patient overall survival in HPV-negative OPSCC, there was a strong correlation within the HPV16-positive OPSCC patient group. These novel findings provide evidence that HPV targets the host cell energy metabolism important for viral life cycle and HPV-mediated tumorigenesis.

In more recent times reprogramming of the energy metabolism has been identified to be one of the hallmarks of carcinogenesis. Increased mitochondrial activity has frequently been associated with unfavorable clinical outcome in a great variety of human cancers, where tumor progression is commonly characterized by an upregulation of glycolysis and impaired mitochondrial activity[1]. This led to the hypothesis of a metabolic switch in cancer cells, a theory which has further been strengthened over the last decades. However, we now know that mitochondrial oxidative phosphorylation (OXPHOS) is not completely abrogated in cancer cells, and that its use may even fluctuate wildly depending on different stages of carcinogenesis, with cancer cells utilizing different energy sources and metabolic pathways to sustain their increased metabolic demands[2]. Although tumor cells cover their energetic demands largely by involving glycolysis, the functional mitochondrial OXPHOS is fundamental not only by providing additional ATP, but also for the production of metabolites that are essential for tumor cell homeostasis and progression. The respiratory chain complexes I–IV maintain a proton gradient, which is required for the generation of ATP by the ATP synthase (F_1F_0-ATPase)[3].

Human papillomaviruses (HPV) are circular double-stranded epitheliotropic DNA viruses with the ability to infect cells of the cutaneous skin as well as the oral or genital mucosa. Such infections may lead to benign but also malign tumors[4]. HPV of genus betapapillomavirus (betaHPV, e.g. HPV8) are known to be associated with skin cancer development, particularly in Epidermodysplasia verruciformis (EV) patients and organ transplant recipients[5–8]. While infections with low-risk HPV types of genus alphapapillomavirus (alphaHPV, eg. HPV6,

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HPV11) only lead to benign genital warts, high-risk alphaHPV, such as HPV16, are the cause for the development of squamous cell carcinoma (SCC) of the anogenital region[9]. Furthermore, HPV16 is also known to frequently cause oropharyngeal squamous cell carcinoma (OPSCC). In contrast to HPV-negative OPSCC, whose main risk factors are smoking and/or alcohol consumption, patients with HPV-positive OPSCC are characterized by better locoregional control and a better clinical outcome[10].

Concerning the oncogenic mechanisms of HPV8, our group previously identified that hyperproliferation and invasion of primary skin keratinocytes is induced by the E7 oncoprotein[11–13]. HPV8-E7-induced invasion of keratinocytes is mainly mediated by an interaction between basal keratinocytes and the extracellular matrix, in particular through the α3β1/fibronectin axis, which we demonstrated in organotypic skin cultures. In the same assays we also identified the $E7^{L23A}$ mutant which was not able to trigger keratinocyte invasion[14]. Several cellular interaction partners of HPV8-E7 have been investigated in previous studies[15,16]. To expand our understanding of how oncogenesis is driven by such interactions, drive oncogenesis, we screened for further E7 interaction partners performing yeast-two-hybrid (Y2H) and Co-IP/Mass-spectrometric (Co-IP/MS) experiments. Here, we identified the mitochondrial protein ATP5B as a potential interaction partner of HPV8-E7. ATP5B is one of the subunits of the mitochondrial ATP synthase, also known as complex V, which is a mitochondrial proton pump[17]. Our results demonstrate that this interaction appears to be crucial for meeting the energy demand required for HPV8-E7-mediated cell proliferation and invasion. Strikingly, the E7 proteins of both HPV11 and HPV16 interact even stronger with ATP5B than HPV8-E7. Lastly, we could show that ATP5B over-expression in HPV16-positive OPSCC correlated with improved patient survival, which was in stark contrast to HPV-negative OPSCC.

Results

Identification of ATP5B as a novel HPV8-E7 binding partner. To identify novel cellular binding partners of HPV8-E7 we performed both Y2H as well as Co-IP/MS approaches using FLAG-tagged E7 transfected C33a cells. Using a cut-off of more than twofold deregulated and $p < 0.05$ we ended up with 289 statistically significant targets out of 315 identified proteins via Co-IP/MS (Supplementary Table S1). Of note, we identified 5 subunits of the mitochondrial ATP synthase, ATP5B, ATP5A1, ATP5C1, ATP5F1 and ATP5L as putative binding partners of E7. Interestingly, in Y2H experiments performed in parallel only ATP5B could be confirmed to be interacting with HPV8-E7 (Supplementary Table S2). Therefore, we decided to focus on this novel HPV8-E7 binding partner, which has—to our best knowledge—not been described in previous studies. To further confirm the interaction of ATP5B with HPV8-E7, we performed additional Co-IP experiments with FLAG-tagged HPV8-E7. In the corresponding Western blots we observed co-immunoprecipitation of ATP5B and HPV8-E7 wildtype, with binding of the invasion defective E7 mutant $E7^{L23A}$ to ATP5B being significantly weaker. Total protein levels of ATP5B were not affected (Fig. 1A,B), which was also confirmed by measuring mRNA expression by means of RT-qPCR for cells grown on either uncoated or fibronectin coated tissue culture plates or differentiated in the presence of 2 mM $CaCl_2$ (Supplementary Fig. S1). To demonstrate that the interaction between HPV8-E7 and ATP5B occurs at the mitochondria, we performed further immunocytochemical staining for the mitochondrial protein Cytochrome C, ATP5B and FLAG-8E7 as ATP5B can be present in both cytosol as well as the mitochondria. Not only did the staining for ATP5B and E7 showed an overlay (Fig. 1C), but also the staining for Cytochrome C and HPV8-E7 showed a distinct overlay (Fig. 1D), indicating that HPV8-E7 and ATP5B co-localize at the mitochondria.

Despite the observation that total protein levels of ATP5B were not altered in vitro, a moderate upregulation of ATP5B was seen in basal and suprabasal cell layers when HPV8-E7 expressing N/TERT keratinocytes were differentiated as organotypic skin cultures. Diminishing signals were detected in upper layers (Fig. 1E). Lastly, we stained normal human skin and EV skin tissues for ATP5B. In normal human skin as well as non-lesional EV skin ATP5B was expression predominantly detected in the basal and suprabasal epidermal layers. In EV tumor tissues, however, ATP5B was ubiquitously, yet weaker expressed throughout the different tumors (Fig. 1F).

AlphaHPV types 11 and 16 bind ATP5B stronger than betaHPV type 8, but do not affect its protein expression levels. Next, we investigated if ATP5B also forms complexes with E7 of alphaHPV types 11 and 16. HEK293 cells were transfected with FLAG-HPV8-E7, FLAG-HPV8-$E7^{L23A}$, FLAG-HPV11-E7 or FLAG-HA-HPV16-E7 and co-immunoprecipation assays were performed with corresponding cell extracts. The results in Fig. 2A show that endogenous ATP5B co-immunoprecipiated with both alpha and beta HPV E7 proteins, however the strength of interactions between HPV11-E7 and HPV16-E7 with ATP5B were stronger than the interactions of HPV8-E7 and HPV8-$E7^{L23A}$ with ATP5B. Since high-risk alphaHPV E7 oncoproteins have been shown to target some of their substrates for proteasomal degradation[18], it was of interest to investigate if this was also the case with ATP5B. To this end, HEK293 cells were transfected with FLAG-HPV8-E7, FLAG-HPV8-E7L23A, FLAG-HPV11-E7 and FLAG-HA-HPV16-E7. After 24 h, the cells were harvested and endogenous ATP5B protein levels were analyzed by Western blotting. Interestingly, the results shown in Fig. 2B demonstrate that both high-risk and low-risk alphaHPV E7 oncoproteins did not have any impact on ATP5B protein stability, despite of their strong interactions with ATP5B. To further examine and confirm this observation, we compared ATP5B protein expression levels in cervical tumor derived HPV18-positive HeLa cells in presence and absence of E6/E7. HeLa cells were transfected with siRNA targeting E6/E7 or with luciferase as a control. Cells were harvested after 72 h, and their ATP5B protein levels were determined by Western blotting. P53 protein was also tested and used as a control for E6/E7 ablation. The results shown in Fig. 2C indicate that there are no noteworthy changes in ATP5B protein levels following siRNA treatment against E6/E7. Taken together, these results suggest that ATP5B is not targeted by alphaHPV E7 oncoproteins for proteasomal degradation.

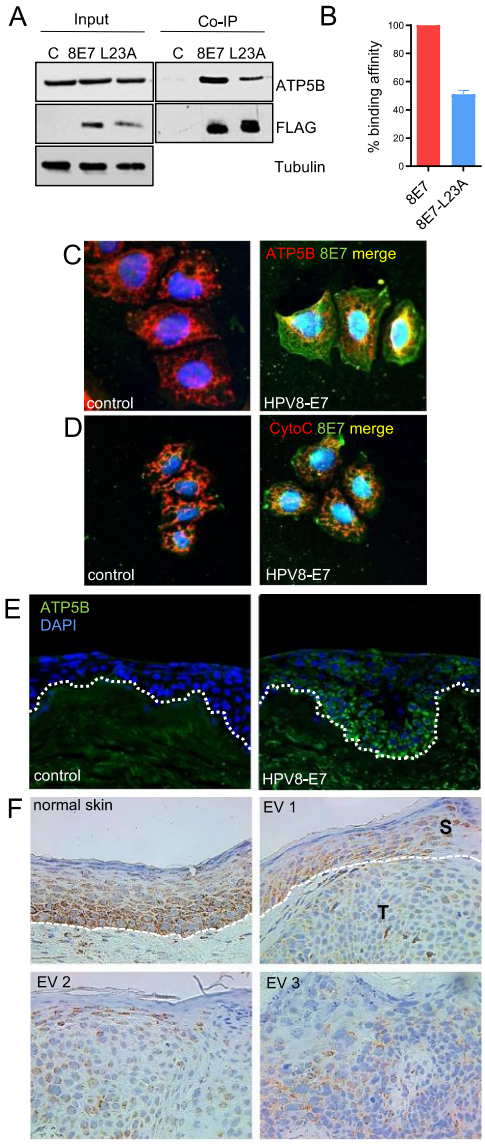

Figure 1. Interaction and co-localization of HPV8-E7 and ATP5B. (**A**) Representative immunoblots of Co-IPs performed with whole protein lysates of C33a cells transiently transfected either with pCMV2-FLAG empty vector, pCMV2-HPV8-E7-FLAG or pCMV-8E7^{L23A}-FLAG. Cell extracts were incubated with M2-FLAG agarose beads. Co-immunoprecipitated ATP5B bound to HPV8-E7-FLAG was detected by immunoblotting with specific antibodies. The expression of HPV8-E7-FLAG was confirmed by a Western blot analysis against FLAG. Equal loading was ensured by staining for tubulin as a control. Images were cropped from different gels for better clarity. Original Western blots presented are available in Supplemental Fig. S2. (**B**) Statistical analysis of n = 4 independent Co-IP experiments. (**C**) C33a cells grown on coverslips were immunocytochemically stained to confirm co-localization of HPV8-E7 and ATP5B (DAPI: blue, ATP5B: red, HPV8-E7: green, overlay: yellow, magnification 600 ×). (**D**) C33a cells grown on coverslips were immunocytochemically stained to confirm localization of HPV8-E7 to mitochondria (DAPI: blue, mitochondria-associated protein Cytochrome C: red, HPV8-E7: green, overlay: yellow, magnification 600 ×). (**E**) Immunofluorescence stainings of organotypic skin cultures of N/TERT keratinocytes either harbouring the empty vector pLXSN (left image) or pLXSN-HPV8-E7 (right image). Elevated ATP5B levels are observed in basal and suprabasal cell layers in the HPV8-E7 culture, and lighter signals in more suprabasal areas (magnification 200 ×). (**F**) Representative immunohistochemical staining of normal human skin as well as non-lesional EV skin and EV skin tumors (EV1/EV2 are positive for betaHPV types 5, 36; EV3 is positive for betaHPV types 5, 8, 20, 23, 36, 50) (magnification 400 ×).

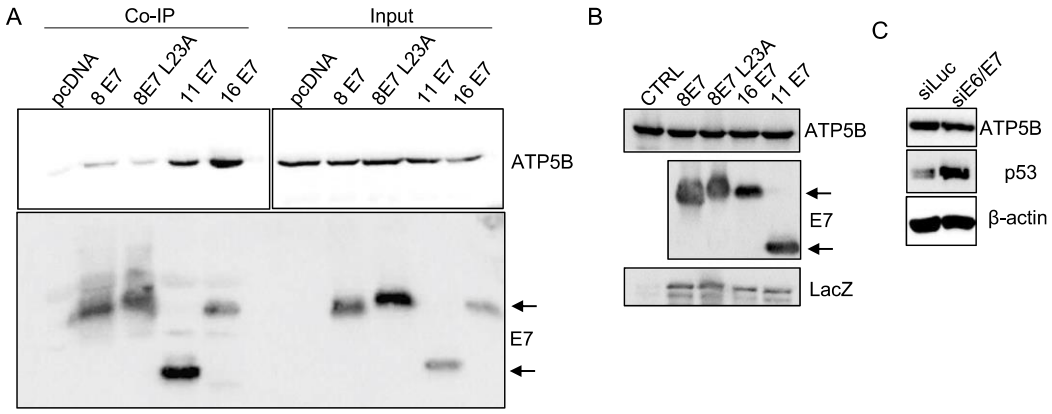

Figure 2. Assessment of ATP5B and E7 binding strengths of HPV11 and HPV16 compared to HPV8-E7. HEK 293 cells were transfected with FLAG-HPV8-E7, FLAG-HPV8-E7L23A, FLAG-HPV11-E7 and FLAG-HA-HPV16-E7. After 24 h cells were harvested and cell extracts were immunoprecipitated with anti-FLAG antibody conjugated beads. Coimmunoprecipitating ATP5B was detected by Western blotting with anti-ATP5B antibody. The protein inputs of ATP5B and E7 are shown. (**B**) HEK 293 cells were transfected with FLAG-HPV8-E7, FLAG-HPV8-E7L23A, FLAG-HPV11-E7 and FLAG-HA-HPV16-E7. After 24 h cells were harvested and residual ATP5B and E7 oncoproteins were detected by Western blot analysis using either anti-ATP5B or anti-Flag antibodies. The expression of β-Galactosidase (LacZ) was used as a control of transfection efficiency. (**C**) HeLa cells were transfected with siRNAs directed against luciferase (siLuc) and HPV18-E6/E7. After 72 h cells were harvested and the levels of ATP5B, p53 (p53 levels were measured to confirm E6E7 knockdown) and the β-actin loading control were detected by Western blotting. Images were cropped from different gels for better clarity. Original Western blots presented are available in Supplemental Fig. S3.

HPV8-E7 and HPV16-E7 reduce glycolytic activity but increase oxidative respiration. To characterize the effect of HPV8-E7 and HPV16-E7 binding to ATP5B we next analyzed the effect of these interactions on glycolytic activity of cells by performing the GlycoStress test on the Seahorse analyzer (Fig. 3A). We show that $^{RM+}$Ntert-8E7, and $^{RM+}$Ntert-16E7 keratinocytes have a significant reduced glycolytic activity regarding the parameters basal glycolysis, glycolytic capacity and glycolytic reserve (Fig. 3B,C). There was no major difference between the different E7 expressing keratinocytes. To conclude, glycolysis is not being utilized by E7 to meet the heightened energy demands of HPV-E7 positive cells.

We next performed MitoStress tests to measure mitochondrial respiration (Fig. 3D). Regarding basal respiration we observed an about two-fold increase in HPV8-E7 cells in their basal respiration compared to control. This effect was elevated to about tenfold in HPV16-E7 keratinocytes. As far as proton leak is concerned, there was no relevant difference between control and HPV8-E7 cells, but there was a statistically relevant rise in proton leak in HPV16-E7 cells. After addition of FCCP the maximum respiration of control cells remained unaffected, whereas a strong increase in maximum respiration was seen in HPV8-E7 keratinocytes, an effect which appeared even more drastic in HPV16-E7 cells. From our gathered data we calculated an about sixfold increase of the SRC in HPV8-E7 keratinocytes, and an about 14-fold increase in HPV16-E7 keratinocytes. (Fig. 3E,F). E7 drastically upregulates mitochondrial activity which is the key energy source in E7 positive keratinocytes in order to meet their spiking energy demands.

ATP5B expression in HPV positive and negative OPSCC. To characterize the ATP5B levels in the presence of HPV16 we next examined a tissue micro arrays (TMA) containing in total 207 patient samples originating from both HPV positive and negative OPSCC. The relevant patient characteristics are described in Supplementary Table S3. Out of these 207 samples 150 samples, with 121 HPV negative, and 29 HPV positive OPSCC could be used for the final analysis. We found a significant correlation between positive HPV status and strong ATP5B expression ($p = 0.011$, Fig. 4A). No such significant correlation was found for T-stage, lymph node metastases, age and gender (Fig. 4A). In line with the observations on OPSCC, we also could detect higher ATP5B levels in high-risk HPV positive cervical cancer tissues ($n = 48$) compared to HPV negative cancers in which no ATP5B is expressed ($n = 6$) (Fig. 4E).

In order to determine whether ATP5B expression levels had an effect on overall patient survival of OPSCC patients, we grouped patients with no or weak ATP5B expression ($n = 14$) and compared them with patients that showed a strong ATP5B expression signals ($n = 15$) (Fig. 4B). We observed that HPV positive tumors with simultaneously high ATP5B expression have a significantly higher overall survival ($p = 0.046$) compared to HPV positive patients with no or very low ATP5B expression (Fig. 4C,D). Curiously, no such association was found in HPV negative patients with no / weak ($n = 88$) or high ($n = 33$) ATP5B expression ($p = 0.921$).

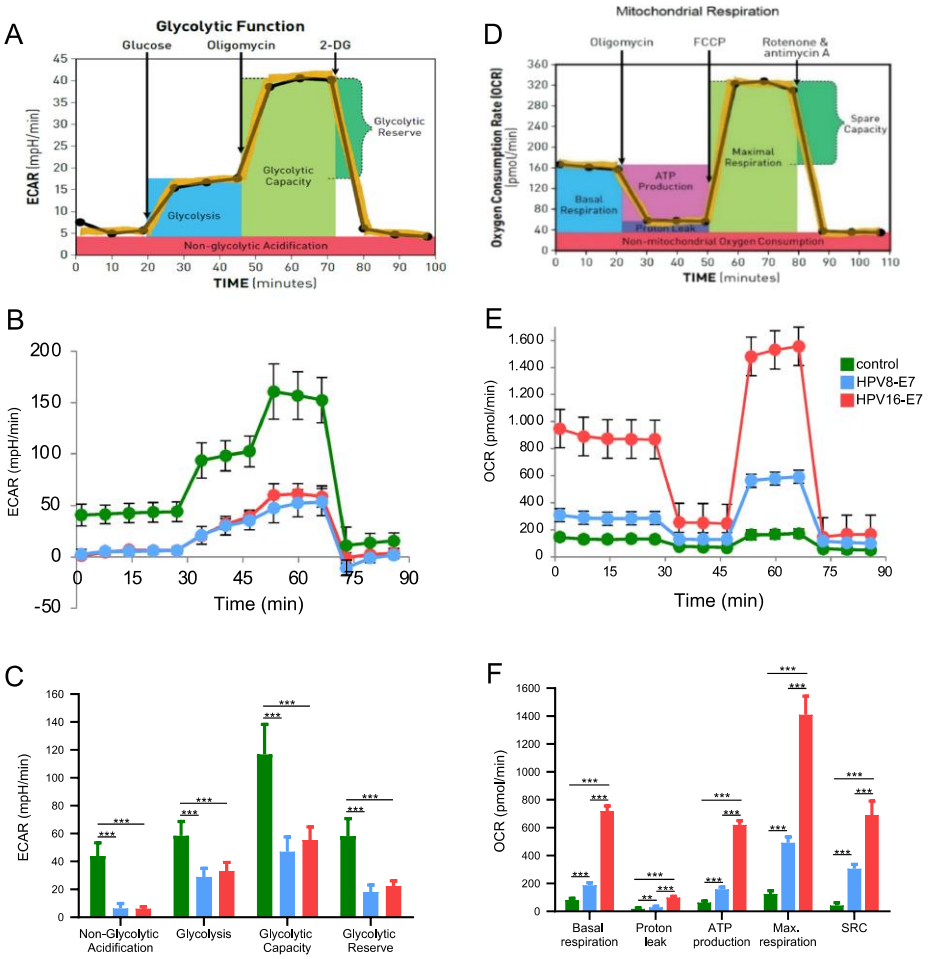

Figure 3. Effect of HPV8-E7 and HPV16-E7 on glycolysis and mitochondrial activity. Schematic principle of the GlycoStress test performed on a Seahorse XF extracellular flux analyzer (**A**) and (**B**) individual parameters for non-glycolytic acidification, glycolysis, glycolytic capacity, glycolytic reserve in N/TERT keratinocytes expressing either the empty retroviral pLXSN, HPV8-E7 or HPV16-E7. Measurements were done in three separate experiments with n = 5 replicates per cell-line. (**C**) Statistical analysis of the individual GlycoStress test parameters (*ECAR* extracellular acidification rate). Schematic principle of the MitoStress test performed on a Seahorse XF extracellular flux analyzer (**D**) and (**E**) individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production in N/TERT keratinocytes expressing either the empty retroviral pLXSN, HPV8-E7 or HPV16-E7. Measurements were done in three separate experiments with n = 5 replicates per cell-line. (**F**) Statistical analysis of the individual MitoStress test parameters (*OCR* O_2 consumption rate, *SRC* spare respiratory capacity).

Discussion

Following HPV infection viral oncoproteins drastically alter the host cell machinery to allow for completion of the viral life cycle which is forcing infected cells into proliferation. This may also extend to invasion of keratinocytes, and thus lead to cancer. It remains unknown how particularly the host cell metabolism is controlled by HPV to meet the increased energy demands.

It has previously been described that high-risk alphaHPV early proteins interact with mitochondria[19]. Most recently it has further been demonstrated, that the E6 protein of high-risk alphaHPVs induces the mitochondrial metabolism by increasing the protein levels of various mitochondrial complexes[20]. Here, we now show that E7 of HPV8, HPV11 and HPV16 all interact with varying affinity with the mitochondrial ATP synthase subunit ATP5B, which may therefore represent a conserved interaction across alpha and beta HPV. Total protein levels were, however, not targeted in vitro. The weaker binding of the invasion deficient mutant HPV8-$E7^{L23A}$ to

A

		(n)	no / weak	(%)	strong	(%)	p[a]
(n)			102	68%	48	32%	
HPV-related	yes	121	14	48%	15	52%	p= 0,011
	no	29	88	73%	33	27%	
T stage	T1-3	112	72	64%	40	36%	p= 0,112
	T4	37	29	78%	8	22%	
N stage	pos	104	71	68%	33	32%	p= 0,848
	neg	45	30	67%	15	33%	
Age (years)	60 <	81	58	72%	23	28%	p= 0,305
	60 >	69	44	64%	25	36%	
Gender	male	121	82	68%	39	32%	p= 0,091
	female	29	20	69%	9	31%	

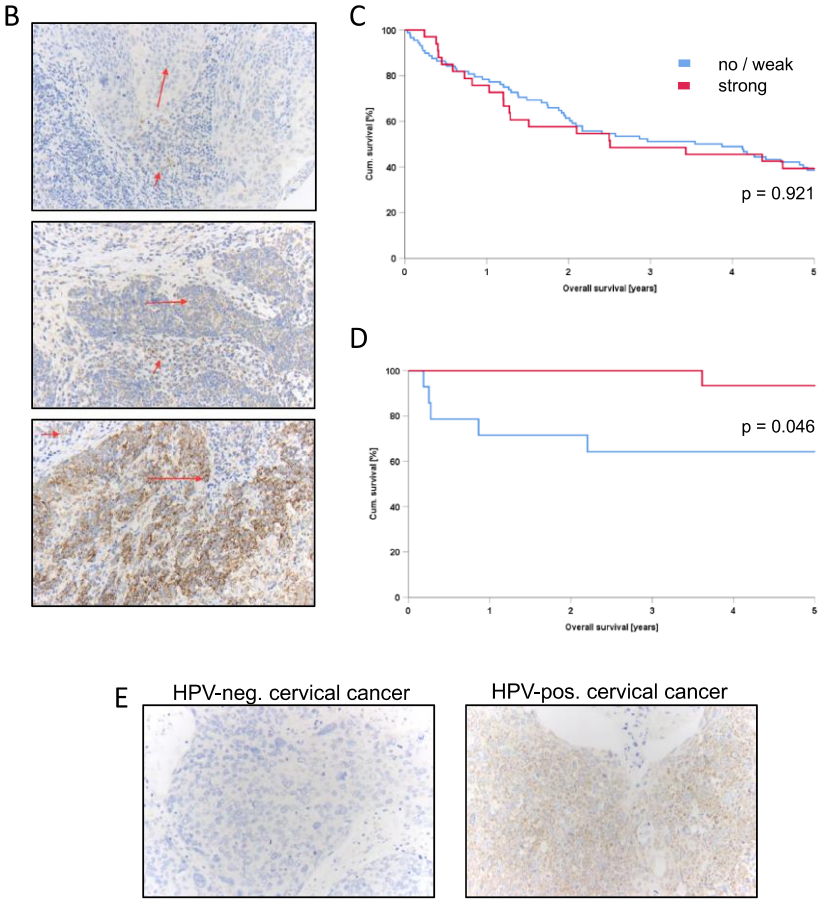

Figure 4. Expression levels of ATP5B in OPSCC and correlation with patient survival. (**A**) Relation of ATP5B expression according to risk factors and tumor/patient characteristics (n = 150). [a]p values calculated by x^2 test (Pearson, asymptotic, two-sided), significant p-values ($p \leq 0.05$) in bold. (**B**) Shown are three immunohistochemical staining intensities. Negative in carcinoma cells (0), weak expression (2 +) and strong expression in carcinoma cells (3 +). The long arrows mark the carcinoma cells, the short arrows the on-slide control in surrounding inflammatory cells (magnification: 200 ×). Overall survival of patients with HPV-negative (**C**) or positive (**D**) OPSCC correlates with ATP5B expression levels (top: HPV-negative; bottom: HPV-positive). (**E**) Representative immunohistochemical staining of ATP5B son HPV negative (n = 6) and high-risk HPV positive (n = 48) cervical cancer tissues.

ATP5B allows for the speculation, that residues around L23 may somehow be involved in binding to ATP5B which may be one possible explanation for why the mutant L23A is inferior in driving proliferation and invasion. An explanation for how the E7 proteins tested in our study enter the mitochondria could be that—as predicted by in silico analysis using MitoFates[21]—they all possess a positively charged amphiphilic region near the N-terminus that may aid in facilitating E7 entry into the mitochondria. In conclusion, ATP5B appears to be a conserved mitochondrial target of alphaHPV and betaHPV in the addition previously described nuclear target proteins. Since the low-risk HPV11 E7 protein binds to ATP5B as well suggests that this event might be required for creating a proliferation-promoting environment essential for completion of the viral life cycle as opposed to carcinogenesis per se.

The observation that E7 interacts with ATP5B sparked the idea that it may exert control over mitochondrial energy production. To this end, we studied both glycolytic activity as well as mitochondrial respiration. We observed, as far as glycolysis was concerned, that it was largely abolished in HPV8-E7 and HPV16-E7 positive cells compared to control cells. This is a curious observation as most tumors re-purpose mitochondria and primarily employ glycolysis to generate the energy required for further tumor progression, particularly in hypoxic regions of solid tumors. Strikingly, HPV8-E7 and HPV16-E7 drastically increased the spare respiratory capacity of mitochondria with HPV16-E7 doing so with much greater efficacy than HPV8-E7. Our metabolic data strongly imply, that HPV infected cells have the ability to react to the rising energy requirements. This might be facilitated by boosting ATP synthase activity, causing it to go into overdrive to generate the required energy for either completion of the viral life cycle, benign or malignant keratinocyte transformation.

In addition to the in vitro experiments, we also characterized ATP5B expression levels in vivo in skin tumors of EV patients (betaHPV positive) and in HPV16 positive and HPV negative OPSCC. In line with the comparatively moderate binding of HPV8-E7 to ATP5B, there was only a moderate elevation in total ATP5B levels in stained OPSCC tissues. However, in accordance with the strong binding affinity of HPV16-E7 to ATP5B we found a significant association of high ATP5B expression in HPV16 positive OPSCC. Curiously, high ATP5B levels positively correlated with overall survival of HPV16 positive OPSCC patients, whereas changes in ATP5B expression played no role in HPV negative OPSCC in respect to their overall survival. However, why the mitochondrial metabolism remains so active in HPV positive cells remains unknown. Previous works have shown that HPV negative head and neck squamous cell carcinoma cells seemingly prefer glycolysis rather than OXPHOS. In contrast, HPV positive HNSCC strongly favor OXPHOS for energy generation[22–25]. Cruz-Gregorio et al. recently showed that HPV16 increases protein levels of subunits of mitochondrial complex I–IV as well as the ATP synthase, leading to increased mitochondrial mass. Furthermore, E6 thus increases basal and leak respiration, which was associated with oxidative stress by increasing the amount of reactive oxygen species without having an effect on ATP-linked mitochondrial activity[20]. From these results the authors concluded that this may partially explain why HPV positive OPSCC are more radiosensitive due to oxidative stress and the resulting DNA damage and the susceptibility to ionizing radiation. Our data regarding E7 further support this theory, as we could show that the mere presence of E7 was enough to both increase ATP production at the expense of higher proton leakage, which may also lead to oxidative stress. This would also explain why HPV positive OPSCC patients with high ATP5B expression have a significantly more favorable clinical outcome. The further characterization of these metabolic changes may therefore be invaluable for the identification prognostic markers or novel drug targets.

Methods

Yeast-2-hybrid. In order to identify cellular proteins binding to HPV8-E7 a Matchmaker two-hybrid system was used together with a HaCaT cDNA library (Clontech, Saint-Germain-en-Laye, France). Experiments were performed according to the manufacturer`s recommendations.

Cell culture. N/TERT keratinocytes[26] were cultivated either in KGM-Gold (containing low (0.05 mM) Calcium) (Lonza, Cologne, Germany) or in RM + medium (consisting of a 3:1 ratio of Dulbecco's modified Eagle's medium [DMEM]-F12 with 10% fetal calf serum [FCS], 1% glutamine, 0.4 µg hydrocortisone, 10^{-10} M cholera toxin, 5 µg/ml transferrin, 2×10^{-11} M liothyronine, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 1 × penicillin–streptomycin mixture)[27]. Retroviral transduction of N/TERT keratinocytes was performed using the retroviral vector pLXSN and the genes encoding for HPV8-E7, HPV8-E7^{L23A} or HPV16-E7 as previously described[14,27].

Organotypic skin cultures based on de-epidermalised human dermis as matrix and retrovirally transduced keratinocytes were generated as previously described[14].

For the generation of FLAG tagged HPV8-E7 or HPV8-E7^{L23A}, the E7 gene was amplified via PCR and cloned into the pCMV2-FLAG vector via the BamHI site. The L23A mutation in HPV8-E7 was introduced by site-directed mutagenesis[14]. The HPV-negative C33A cell line was cultured in DMEM, supplemented with 10% FCS and 1% Penicillin/Streptomycin. C33A cells were transfected in 6-wells using the $CaCl_2$-method with either the empty expression vector pCMV2-FLAG or pCMV2-HPV8-E7-FLAG, pCMV2-HPV8-E7^{L23A}-FLAG constructs. HEK-293 cells were transfected in 10 cm^2 dishes using the $CaCl_2$ method either with the empty expression vector pCMV2-FLAG or pCMV2-HPV8-E7-FLAG, pCMV2-HPV8-E7^{L23A}-FLAG, Flag-HA-tagged pCMV HPV-16 E7 and FLAG-tagged pCMV HPV-11 E7[28]. siRNA transfections were performed as previously described[29]. In brief, HeLa cells (HPV18 positive) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and penicillin/streptomycin. HeLa cells were seeded in 6 cm^2 dishes and transfected using DharmaFECT transfection reagent (Horizon Discovery, Germany) according to the manufacturer's instructions with siRNA against luciferase (Horizon Discovery) as the control and siRNA against 18E6/E7 (5′ CAUUUACCAGCCCGA CGAG) (custom ordered form Horizon Discovery).

Co-IP. 10 µl of α-FLAG M2 affinity resin (Sigma-Aldrich, Taufenkirchen, Germany, A229) were equilibrated with 0.1 M LSDB buffer (1 mM DTT, 1 mM PMSF) for each sample and centrifuged at 7000 rpm for 2 min at 4 °C. The beads were resuspended in 85 µl 0.1 M LSDB and transferred into a new tube. Then, 300–500 µg of whole protein lysate containing FLAG-fusion proteins were added to the beads, and the mixture was filled up to 1 ml with 0.1 M LSDB, before incubating them on a rotator for 3 h at 4 °C. This step was followed by centrifuging at 7000×*g* for 5 min at 4 °C. Afterwards, supernatants were discarded and samples were washed five times in 1 ml 0.1 M LSDB, before reconstituting them in 30 µl of 0.1 M LSDB. Then, 10 µl of 4 × SDS loading puffer were added and samples were incubated for 5 min at 95 °C. After centrifuging the samples at 7000×*g* for 5 min the supernatants were separated by means of SDS–PAGE and subsequent Western Blots, probing for ATP5B (Sigma Aldrich, HPA001520) or FLAG (Sigma-Aldrich, F4042).

Western blot. Western blots were performed as described previously[30]. Briefly, cell pellets were resuspended in RIPA buffer containing protease inhibitors, incubated on ice for 30 min, sonicated and then centrifuged at 15,000×*g*, at 4 °C for 15 min. Protein concentrations were measured with the PierceTM BCA Protein Assay Kit (ThermoFisher, Dreieich, Germany). SDS Gels were transferred upon completion to nitrocellulose membranes, which were then blocked with 5% dry milk in TBST. The primary antibodies used targeted ATP5B (HPA001520, Sigma Aldrich), FLAG (F4042, Sigma-Aldrich), anti-p53 (DO-1, Santa Cruz Biotechnology), β-actin (Sigma-Aldrich), β-galactosidase (LacZ, Promega) or tubulin (ab6160, Abcam, Cambridge, UK) and incubated for 2 h at RT or overnight at 4 °C. Following washing, membranes were incubated with corresponding secondary antibodies, after which the membranes were developed using BM Chemiluminescence blotting substrate (Sigma-Aldrich). Western Blots were either visualized using an ECL-hyperfeature film (Amersham, Freiburg, Germany) or by employing the GelDoc system from BioRad (Feldkirchen, Germany).

Sample preparation for mass-spectrometry. Co-immunoprecipitated cellular proteins bound to HPV8-E7-FLAG were processed and analyzed at the CECAD Proteomics facility, University Hospital Cologne. Afterwards in-solution digest of proteins was performed with reagents provided by the facility: 50 × Protease Inhibitor cocktail (Roche), triethylammoniumbicarbonate (TEAB), 50 mM, urea buffer: 8 M Urea in 50 mM TEAB, benzonase HC nuclease , dithiothreitol (DTT), chloroacetamide, trypsin protease, 1 µg/µL or 0.1 µg/µL, lysyl Endopeptidase (Lys-C), 0.5 µg/µL , formic acid, 10% in water. Firstly, urea lysis buffer was prepared by adding 50 × protease inhibitor to 8 M Urea/50 mM TEAB buffer. Protein lysis was achieved by resolving Urea at 4 °C on a rotator using the concentrated supernatants as solvent to arrive at a final concentration of 8 M. Afterwards samples were centrifuged for 15 min at 20,000×*g* to remove any cell debris. Afterwards protein concentration was measured and 400 µg per sample were transferred to a new 1.5 ml Eppendorf tube. To reduce background noise for the mass spectrometric analysis a sample containing only medium was included. Afterwards DTT was added to each sample at a final concentration of 5 mM, briefly vortexed and then incubated at room temperature for 1 h. Afterwards CAA was subsequently added at a final concentration of 40 mM, the sample vortexed and incubated in the dark for 30 min, before adding Lys-C protease at an enzyme:substrate ratio of 1:75, followed by another incubation period of 4 h at 25 °C. Following this step samples were diluted with 5 mM TEAB to achieve lower urea concentration of 2 M. Then trypsin was added at an enzyme:substrate ratio of 1:75 and the samples were incubated at 25 °C overnight. The following day samples were acidified by adding 1% formic acid to stop the enzymatic reaction. Afterwards, proteins within the supernatants were desalted and further purified using in-house-packed seppack stage-tipps (Waters GmbH, 65760 Eschborn, Germany). The columns were primed with 1 × 1 ml 100% acetonitrile, followed by three washing steps with three times with 3 ml 0.1% formic acid. Afterwards the supernatants were loaded onto stage tip purification columns, which were then washed three times with 3 ml 0.1% formic acid. The stage tips were then transferred to new Eppendorf tubes and proteins were eluted with 300 µl acetonitrile/trifluoroacetic 0.1% acid per sample. Afterwards the eluates were concentrated using a speed vac prior to mass spectrometric analysis.

Mass-spectrometry. Mass-spectrometric analysis was performed as recently described[31]. For tandem mass spectrometry LC–MS/MS analysis (LC–MS/MS or MS2) and easy nLC 1000 (Thermo Scientific) were coupled to the quadrupole-based Q Exactive Plus (Thermo Scientific) instrument using a nano-spray ionization source. All mass spectrometric proteomics raw data were analyzed using the bioinformatics tools MaxQuant (v1.5.3.8) running with default parameters and Perseus (v1.5.0.3.1). Briefly, MS2 spectra were searched against the Uniprot HUMAN.fasta (downloaded at: 16.6.2017) database, including a list of common contaminants. False discovery rates (FDR) on protein and post-translational modification (PSM) levels were evaluated by a target-decoy approach to 1% (Protein FDR) and 1% (PSM FDR) respectively. The minimal peptide length was set to seven amino acids and carbamido-methylation at cysteine residues was considered as a fixed modification. Oxidation (M) and Acetylation (Protein N-term) were included as variable modifications and match-between runs option was enabled. LFQ quantification was enabled using default settings.

Seahorse assay for measurement of glycolytic stress. Measurement of cellular glycolytic activity was performed using the Seahorse XF96e analyzer (Agilent Technologies, Massachusetts, USA). N/TERT-HPV8-E7, N/TERT-HPV16-E7 and control cells were grown in RM + medium and seeded out on Seahorse XF96 cell culture microplates 16 h prior to measurement at a cell density of 25,000 cells per well. Assay medium was prepared on the day of the assay by supplementing Seahorse XF Base Medium with 2 mM glutamate and no other additives. Then the medium was warmed to 37 °C and the pH adjusted to 7.4. Forty-five minutes prior to measurement cell culture medium was replaced by assay medium, followed by adding 5 mM glucose, 1 µM Oligomycin and 100 mM 2-Deoxy-D-glucose (2-DG) at different time-points. Following measurement of glycolytic

activity protein concentration was determined including a standard curve. All cell lines were at least measured n = 10 times and normalized to total protein content, including a BSA standard with known protein concentrations. The data was later analysed using the Seahorse Report Generator.

Seahorse assay for measurement of mitotic stress. Measurement of cellular respiration was performed using the Seahorse XF96e analyzer as previously described[32]. Cells were grown in either KGM Gold or RM + medium and seeded out as described above. Prior to respiration assays, cell culture medium was replaced by prewarmed, pH 7.4 adjusted assay medium supplemented with 5 mM glucose, 10 mM sodium pyruvate and 2 mM glutamate according to the manufacturer's protocol. Oxygen consumption rate (OCR), extracellular consumption rate (ECAR) and proton production rate were measured under basal conditions and in the presence of oligomycin, a complex V inhibitor (1 µM), the complex III inhibitor antimycin A (0.5 µM), the complex I inhibitor rotenone (0.5 µM) and the mitochondrial uncoupler carbonyl-cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1.5 µM) to assess maximal oxidative capacity. Following the respiration assay, media was removed, wells were washed once with PBS and total protein concentration was measured using the DCTM Protein Assay Kit II (BioRad). All cell lines were at least measured n = 10 times and normalized to total protein content, including a BSA standard with known protein concentrations. The data was later analysed using the Seahorse Report Generator. Spare respiratory capacity (SRC) was defined as the difference between basal and maximum respiration.

Immunocytochemical staining. Staining was performed as described recently Cells were seeded in a 24-well plates at 2×10^4 cells per well on coverslips and cultured for one day. Afterwards cells were washed once with PBS, then fixed with either 4% Formaldehyde for 15 min at RT or with ice-cold 1:1 Aceton/Methanol for 10 min at – 20 °C. In this case cells were washed two times with PBS afterwards. Following fixation, cells were permeabilized by incubating them with 0.5% Triton X-100 for 10 min. Afterwards the cells were washed twice with PBS. To block unspecific binding sites, cells were incubated for 1 h at RT with blocking solution (10% goat serum, 0.1% Tween-20 diluted in PBS) for 1 h. Primary antibodies targeting ATP5B (HPA001520, 1:100 dilution, Sigma Aldrich), FLAG (F4042, 1:2000 dilution, Sigma-Aldrich) or Cytochrome C[33] were diluted in PBS containing 1% goat serum/0.1% Tween. After incubation over night at 4 °C, plates were washed with PBS, and fluorescently labeled secondary antibodies were added to each well diluted in PBS (1% goat serum/0.1% Tween, 1:500 dilution). Afterwards cells were washed twice with PBS, and then incubated with 1 µg/mL 4′,6-diamidino-2-phenylindole (DAPI, diluted 1:1000) for 5 min. Afterwards, coverslips were mounted onto coverslides with Immunomount (Fisher Scientific, Schwerte, Germany) for analysis. Fluorescence images were acquired on a Leica DMI 6000B microscope equipped with a Leica DFC365 FX camera and then analyzed with the Leica LAS X imaging software (v3.3.0.16799).

Preparation of tissue microarrays. Tissue Microarray (TMA) construction was performed as previously described[34,35]. In brief, tissue cylinders with a diameter of 1.2 mm each were punched from tumor tissue blocks using a self-constructed semi-automated precision instrument and embedded in empty recipient paraffin blocks. Four µm sections were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ) for mRNA detection and immunohistochemistry. After IHC staining, 196 of 207 OPSCC tissue cylinders included in TMA blocks were suitable for evaluation after IHC staining.

Immunohistochemical staining. Staining procedures were described previously[30,36]. Formalin-fixed, paraffin-embedded sections were deparaffinized in 100% xylene and rehydrated in decreasing concentrations of ethanol (100%, 90% and 70%). Antigen unmasking was performed by boiling tissue sections in 10 mM citric buffer for 3 min, followed by 15 min resting at RT. Blocking of unspecific antigen sites was achieved with 50% goat serum in PBS for 1 h at RT. Organotypic skin sections were incubated over night at 4 °C with antibodies targeting ATP5B (ab14730, 1:100 dilution, Abcam). Following extensive washing, sections were incubated with secondary antibodies conjugated with Alexa Fluor 488 (A-11029, Life Technologies, Carlsbad, CA, USA) for 1 h at RT. After counterstaining with DAPI, fluorescence images were acquired with a Leica DMI 6000B microscope equipped with a Leica DFC365 FX camera and analyzed with Leica LAS X imaging software (v3.3.0.16799). Immunohistochemical staining of formalin-fixed and paraffin-embedded OPSCCs were performed on tissue micro-arrays (TMA) slides using an automated Leica Bond Stainer. Lymphoid tissue served as an internal control. For TMAs, one tissue core from each tumor was punched out and transferred into a TMA recipient block. In brief, tissue cylinders with a diameter of 1.2 mm each were punched from selected tumor tissue blocks using a self-constructed semi-automated precision instrument and embedded in empty recipient paraffin blocks. Four-micrometer sections of the resulting TMA blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ, USA) for immunohistochemistry.

Reverse transcription quantitative-PCR (RT-qPCR). RT-qPCR was performed to quantify mRNA levels of cellular genes using a LightCycler system (Roche Diagnostics) as previously described[37]. The primers used were: ATP5B-fw 5′: GTTGGGGTTTGTGGGTCGGGTG; ATP5B-rev 5′: TTTGGCGAAGGAGATGTT TGCG; HPRT1-fw 5′: TGACACTGGCAAAACAATGCA; HPRT1-rev 5′: GGTCCTTTTCACCAGCAAGCT. ATP5B expression was normalized to HPRT1 expression levels and the control was set to 1 (n = 4 measurements).

Patients, tumor samples and clinical data collection. Normal human skin was obtained from the Department of Dermatology of the University upon informed consent from all the subjects. Ethical approval was

obtained from the ethics Committee at the University of Cologne, Germany (registration no.: 08-144). EV lesions (n = 9) were obtained from paraffin blocks removed during routine surgical excisions (for detailed information on betaHPV typing and pathology results of EV lesions, see[38]). The use of EV skin tumors for scientific studies was approved by the ethics committee of the Medical University of Warsaw, Poland. Tissue-micro-array (TMA) specimens of formalin-fixed, paraffin-embedded cervix carcinomas were obtained from the archive files of the Department of Pathology, University of Cologne. Clinical information was obtained from the patients' medical records. The collection of cervical cancer tissues was realized according to BioMaSOTA votum (approval number 13-091) at the University of Cologne, Germany. Patients diagnosed with OPSCC between 2000 and 2009 and with sufficient tumor tissue available were included in this study. Written, informed consent for medical and scientific purpose was obtained from all OPSCC patients and tumor material was used in accordance with the regional ethics committee in Giessen, Germany (AZ 95/15, dated 19th October, 2015). All methods were performed in accordance with the relevant guidelines and regulations. Patients were treated according to local guidelines at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery of the University of Giessen. Available formalin-fixed, paraffin-embedded (FFPE) cancer tissue with a thickness of 2–3 mm was mandatory to produce TMA cores. Therefore, patients treated with primary chemoradiation were frequently excluded since diagnostic tumor samples were usually insufficient in size for TMA preparation. FFPE samples of 207 primary cancers of the oropharynx (C09, C10, International Classification of Diseases for Oncology (ICD-O)) were embedded in TMA blocks. Tumor staging and histological grading was assessed according to the International Union against Cancer (UICC) TNM classification (2002)[39] and the WHO criteria for squamous cell carcinomas of the oral mucosa[40].

HPV-DNA genotyping and p16^{INK4a} Immunohistochemistry. HPV-status was determined retrospectively as described[41,42]. In brief, DNA was extracted from variable numbers of FFPE tissue sections depending on the tissue size (10 µm sections, approximately corresponding to 10 × 10 mm tumor tissue) using the DNeasy Blood and Tissue Kit by Qiagen, Hilden, Germany, according to manufacturer's instructions. Extracted DNA was analyzed for mucosal high-risk HPV-DNA and HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) as previously described[43,44]. p16^{INK4a} expression was detected using the CINtec Histology kit (Roche mtm Laboratories, Mannheim, Germany) according to antibody suppliers' and standard protocols[45].

Statistical and survival analysis. Statistical analyses were performed using SPSS statistical software (IBM SPSS 25.0, Chicago, Illinois, USA). Overall survival of OPSCC patients was calculated from initial date (date of histological diagnosis by routine biopsy) to date of death. Follow-up times of event-free patients were not censored. OS rates were calculated by the Kaplan–Meier method. Statistical significance of differences was calculated by log-rank test and chi-square test as appropriate. P-values ≤ 0.05 were considered significant for all tests[46].

All in vitro experiments were repeated a minimum of three times. RT-qPCR data were expressed as mean ± sd. The data presented as immunoblots or images of immunohistochemical analysis are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with unpaired 2-tailed Student's t-test. The asterisks shown in the figures indicate significant differences between experimental groups (**$p < 0.01$, ***$p < 0.001$)[30].

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Author contributions

B.A. designed and supervised this study and drafted the manuscript. M.K., S.H., G.P.M., M.H., J.M.S., A.D., A.Q. performed the experiments. S.M., S.W., C.W., J.P.K. collected tissue samples and the clinical data. M.K., V.T., N.W., H.K., A.Q., B.A. analyzed and interpreted the data. All the authors read and approved the final manuscript.

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Own substantial contributions to this publication:

- Performed immunofluorescence stainings against CHK1 and LC3B on organotypic skin cultures expressing HPV8-E6, E7 or E6/E7 as shown in Fig. 5;
- Contributed to manuscript preparation for submission and revised the paper in response to reviewer comments.

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Human papillomavirus type 8 oncoproteins E6 and E7 cooperate in downregulation of the cellular checkpoint kinase-1

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Human papillomavirus 8 (HPV8) is associated with the development of squamous cell carcinoma (SCC) of the skin. HPV-infected keratinocytes are able to override normal checkpoint control mechanisms and sustain cell cycle activity, allowing for synthesis of cellular proteins necessary for viral genome amplification. To study how HPV8 may disrupt cell cycle control, we analyzed the impact of HPV8 early gene expression on one of the key regulators of cell cycle and DNA damage response, checkpoint kinase-1 (CHK1). We found that expression of E1, E1^E4, E2, E6 or E7 individually did not affect CHK1; however, keratinocytes expressing the complete early genome region (CER) of HPV8 showed a profound loss of CHK1 protein levels, that proved to be mediated by E6E7 co-expression. Neither CHK1 promoter regulation nor the ubiquitin-proteasome pathway are involved in HPV8-mediated CHK1 repression. However, CHK1 protein repression in organotypic skin cultures was paralleled by downregulation of the autophagy marker LC3B. Treatment of HPV8-CER expressing cells with the autophagy inhibitor Bafilomycin A1 rescued CHK1 expression and led to LC3B accumulation. Taken together, our data implicate that CHK1 autophagic degradation is enhanced by HPV8, which may contribute to the oncogenic potential of the virus.

Introduction

Human papillomaviruses (HPV) infect proliferating basal cells of cutaneous or mucosal epithelia. The productive phase of the life cycle is restricted to differentiated suprabasal cells.[1] To ensure an efficient and reproductive life cycle, HPVs finely modulate the activity of cellular proteins and their own viral gene products through various mechanisms. While normal epithelial cells exit the cell cycle upon differentiation or UV-induced DNA damage, HPV-infected cells are able to circumvent the governing checkpoint mechanisms and force cells to remain in the cell cycle, allowing for synthesis of cellular proteins necessary for viral genome amplification.[2,3] The pathogenic effects of HPV in general result from an interplay of viral early proteins, either by exerting control over cellular gene expression, inactivation through direct binding of cellular proteins, or targeted protein degradation by the cell host proteome.[4,5]

The oncogenic potential of HPV of the genus betapapillomavirus (betaHPV) in skin carcinogenesis was originally identified in patients suffering from the rare inherited disease epidermodysplasia verruciformis (EV), who have an increased susceptibility to betaHPV infections. HPV5 and HPV8, both members of the beta 1 species, have been found in 90% of cutaneous squamous cell carcinoma (SCC) in EV patients.[6,7] However, betaHPV can also be found in skin cancers of non-EV patients. Especially, immunosuppressed organ-transplant-recipients also have a higher susceptibility to betaHPV infections of the skin as well as an increased risk of developing SCC compared to healthy individuals.[8,9] The betaHPV DNA loads in actinic keratoses exceed those in SCC, which suggests a particular involvement of betaHPV in the early stages of cutaneous oncogenesis.[10]

To unravel the molecular mechanisms underlying HPV8-mediated skin tumor development, we previously expressed the complete early genome region (CER, inducing simultaneous expression of E6, E7, E1, E1^E4, E2) in primary human adult keratinocytes. Interestingly, we observed irregularly shaped cells, with larger cell size and complexity in comparison to cells individually expressing E2, E6 or E7, respectively.[11] In addition, transgenic mice expressing HPV8-CER under the control of the keratin-14 promoter (K14-HPV8-CER) developed spontaneous as well as UV-induced skin papillomas with partial dysplasia, which in approximately 6% even led to the formation of skin SCC.[12–14] These tumors resulted from HPV8 oncoprotein

Key words: betapapillomavirus, HPV8, E6E7, CHK1, autophagy
Abbreviations: betaHPV: betapapillomavirus; CER: complete early genome region; CHK1: checkpoint kinase-1; HPV: human papillomavirus; LC3B: microtubule-associated protein light chain 3B
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What's new?
While human papillomavirus type 8 (HPV8) is suspected of playing a role in cutaneous squamous cell carcinoma, the contributions of HPV8 to skin tumor development remain unknown. Here, expression of the complete early genome region of HPV8 was associated with a significant reduction in checkpoint kinase-1 (CHK1) protein levels. CHK1 downregulation by HPV8 was dependent on E6E7 oncoprotein co-expression and autophagic processes. The data demonstrate a novel oncogenic effect of HPV8 in keratinocytes, whereby CHK1 absence enables virus-infected cells to survive in a replicative state, predisposing them to the accumulation of DNA damage from ultraviolet radiation.

mediated prevention of UV-induced DNA damage repair and overriding physiological cell cycle checkpoints, thus allowing damaged cells to persist and even to replicate whilst harboring DNA lesions.[15,16] In addition, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related protein (ATR), both key proteins of the DNA damage repair pathway, are decreased in cells expressing HPV5 or HPV8 E6 proteins in a p300-dependent manner.[17–19] A key cell cycle and DNA damage response regulator is the serine/threonine checkpoint kinase-1 (CHK1). It is activated *via* ATR by phosphorylation in response to DNA strand breaks or replication stress. CHK1 in turn inactivates the phosphatase cell division cycle 25 (CDC25), leading to cell cycle arrest. RNAi-mediated knockdown of CHK1 in human cells revealed its essential role in controlling CDC25A protein turnover, and thus controls both normal S-phase entry and the intra-S phase DNA damage checkpoint.[20,21] Previous studies confirmed a requirement for CHK1 in the G2/M checkpoint in response to ionizing radiation,[22] including DNA-damaging drugs.[23] Of note, ATR-dependent Ser317 and Ser345 phosphorylation of CHK1 deliver a signal that not only activates CHK1 but also marks it for ubiquitin-dependent proteasomal degradation,[24–26] a pathway that is also activated in normal cells in response to replicative stress.[27] Furthermore, autophagy was recently discovered as another CHK1 degradation pathway.[28] More recently, our own group could show that HPV8-E6 was sufficient to inhibit ATR and CHK1 phosphorylation. Curiously, total protein levels in monolayer cell-culture following UV irradiation remained unchanged.[16]

Given the importance of CHK1 in genome surveillance pathways, we analyzed whether total CHK1 levels might be altered by simultaneous expression of all early HPV8 proteins. Here, we provide evidence that simultaneous expression of the HPV8 oncoproteins E6E7 leads to a decrease of total CHK1 protein levels involving autophagic processes.

Material and Methods

Cell culture

Primary human adult keratinocytes (PHK) were isolated from discarded abdominal skin, which were obtained with informed written consent from patients from the dermatology clinics at Barts and The London NHS Trust. Ethical approval was granted by the East London and City local research ethics committee. Primary human keratinocytes were isolated, propagated on lethally irradiated NIH3T3 feeder cells and grown in RM+ medium (consisting of a 3:1 ratio of Dulbecco's modified Eagle's medium [DMEM] and Ham's F-12 with 10% fetal calf serum [FCS], 1% glutamine, 0.4 μg hydrocortisone, 10^{-10} M cholera toxin, 5 μg/mL transferrin, 2×10^{-11} M liothyronine, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, 1× penicillin–streptomycin mixture). The spontaneously immortalized human skin keratinocyte cell line PM1[29] and the cancer cell line RTS3b[30] were cultured in RM+ media. The NIH3T3-mouse fibroblast line PT67 (Clontech, Heidelberg, Germany), cultured in DMEM, supplemented with 10% FCS and antibiotics, was used to replicate amphotropic retroviruses. To block proteasomal degradation, PM1 cells were cultured in RM+ medium supplemented with 50 μM MG132 (C2211, Sigma-Aldrich, Taufkirchen, Germany) for 4, 8 and 24 h or for 24 h with DMSO. Bafilomycin A1 treatment (SML1661, Sigma-Aldrich) was performed for 4 h before cell harvest at a final concentration of 100 nM.

Generation of plasmids and production of recombinant retroviruses and infection of keratinocytes with retroviruses

The pLXSN based retroviral constructs, encoding for HPV8-E2, −E6, −E7, E6E7 or CER have previously been described.[11,31–33] A pBabe based vector encoding for E1^E4 was generated by PCR with the following primers: fw: CATGGATCCGCGATGGCGG ATCATAAAGCTCC and rev: CATGAATTCCGGTTACTGG GGGGTCGATAGC, and subsequent insertion of the coding gene region into the BamHI/EcoRI restriction sites.

ORF mutants in the context of the pLXSN-HPV8-CER plasmid were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions using the following primers, leading to a stop codon (*) in the respective gene without affecting the overlapping ORF.

E1-A978T leading to MADHKGSTS*:
CER-E1mut-fw:
GGTAGTACATCTTAAGAAGGGTTAAGTGAGTGG;
CER-E1mut-bw:
CCACTCACTTAACCCTTCTTAAGATGTACTACC;
E1^E4-A963T leading to MADH*:
CER-E1^E4mut-fw: CTGCAAACATGGCGGATCATTAAGG TAGTACATCTAAAGAAG;
CER-E1^E4mut-bw: CTTCTTTAGATGTACTACCTTAATG ATCCGCCATGTTTGCAG;
E2-G2782 T leading to MENLSERFNVLQDQLMNIYEAAE QTL*:

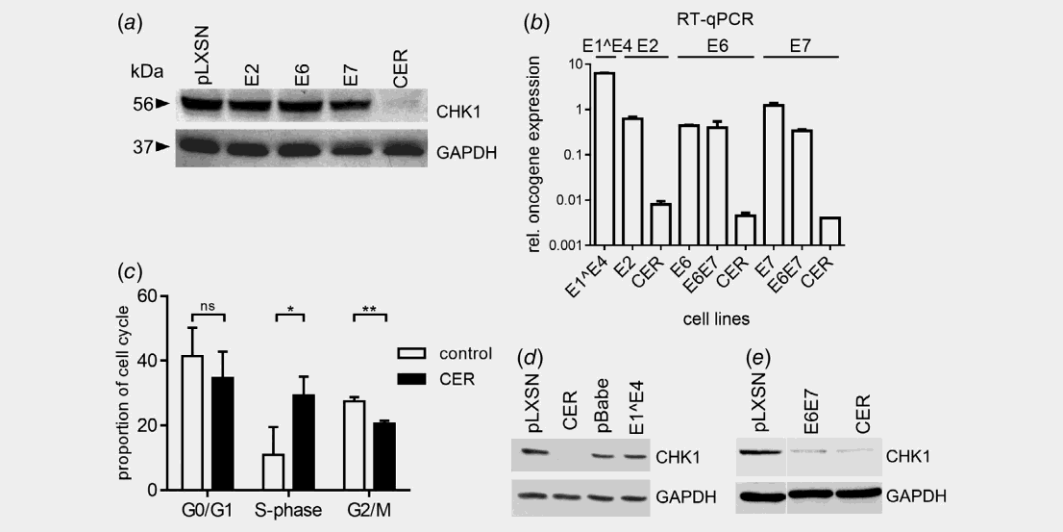

Figure 1. Keratinocytes expressing HPV8-CER exhibit CHK1 depletion. (*a*) Representative immunoblots of RIPA extracts from PHK expressing HPV8 early proteins tested for CHK1 protein expression ($n = 3$). Equal loading was confirmed by immunoblotting for GAPDH. (*b*) Expression of HPV8 oncogenes in different PM1 cell lines was confirmed by RT-qPCR ($n = 2$ experiments in duplicates). Oncogene expression was normalized to the expression of the housekeeping gene HPRT1. (*c*) Flow cytometric analysis of control and HPV8-CER expressing PM1 cells treated with BrdU and PI. Cells were gated for G0/G1, S- and G2/M-phase of the cell cycle. Bars show percentage of gated cells ± SD of $n = 3$ independent experiments. Representative immunoblots of RIPA extracts from PM1 cells expressing (*d*) E1^E4 or (*e*) E6E7 or HPV8-CER were tested for CHK1 protein expression ($n = 3$). Equal loading was confirmed by immunoblotting for GAPDH.

CER-E2mut-fw:
CAGAACAAACACTTTAGGCACAGATTGCGC;
CER-E2mut-bw:
GCGCAATCTGTGCCTAAAGTGTTTGTTCTG;
E6-C205T leading to MDG*:
CER-E6mut-fw: CCTAAGCAAATGGACGGGTAGGACAAG GCTTCATATTTAG;
CER-E6mut-bw: CTAAATATGAAGCCTTGTCCTACCCGT CCATTTGCTTAGG;
E7-G665 T leading to MIGK*:
CER-E7mut-fw:
CATGATTGGTAAATAGGTCACTGTGCAAGATTTTGTG;
CER-E7mut-bw:
CACAAAATCTTGCACAGTGACCTATTTACCAATCATG;

Recombinant retroviruses were produced by transfecting the plasmids into PT67 cells with FuGene 6 (E2691, Promega, Mannheim, Germany), following manufacturer's instructions. PHK at passage 1 were seeded out in Defined Keratinocyte-serum free medium (SFM) (Thermo Fisher, Darmstadt, Germany) at a cell density of 9×10^4 cells in 6 cm dishes. Transduction of PHK with recombinant retroviruses was performed as described before.[34,35] Two days after transduction, cells were seeded out in selection media containing 500 µg/mL G418 for up to 5 days. Resistant cells were grown to confluence, followed by collecting the retrovirus-containing supernatants. Infection of PM1 cells was performed as described before.[36,37]

RNA isolation, reverse transcription and real-time PCR

To quantify mRNA levels of cellular and viral genes, quantitative reverse transcription-PCR (RT-qPCR) using the LightCycler system (Roche, Mannheim, Germany) was performed as previously described.[34] The primers used for RT-qPCR were: GAPDH-fw: TGCACCACCAACTGCTTAGC and GAPDH-rev: GGCATGGACTGTGGTCATGAG[38]; CHK1-fw: TGACTTCCGGCTTTC TAAGG and CHK1-rev: TGTGGCAGGAAGCCAAAC; HPR T1-fw: TGACACTGGCAAAACAATGCA and HPRT1-rev: GGTCCTTTTCACCAGCAAGCT; E1^E4-fw: GGCGGATCAT AAAGCTCCA and E1^E4-rev: GTCTGGACGTGGAGTCAGC; HPV8E2-fw: AACAGCCACAACAAACCG and HPV8E2-rev: AGGACCTGGACCTGGATACG; HPV8E6-fw: GCAACGTTT GAATTTA and HPV8E6-rev: CATGATACAAATGCTTAC; HPV8E7-fw: CCTGAAGTGTTACCAGTTGACCTGC and HP V8E7-rev: CAGTTGCGTTGACAAAAAGACG.

Western blotting

Western blot analyses were carried out as previously described.[39] The blots were probed with antibodies targeting CHK1 (clone G-4, sc-8408, Santa Cruz, Heidelberg, Germany) or mono- and polyubiquitinylated conjugates (clone FK2H, PW0150, Enzo Life Sciences GmbH, Lörrach, Germany). Antibodies for GAPDH (ab9484, Abcam) and tubulin (ab6160, Abcam) were used as loading controls. Immunoreactive proteins were visualized using peroxidase-coupled secondary

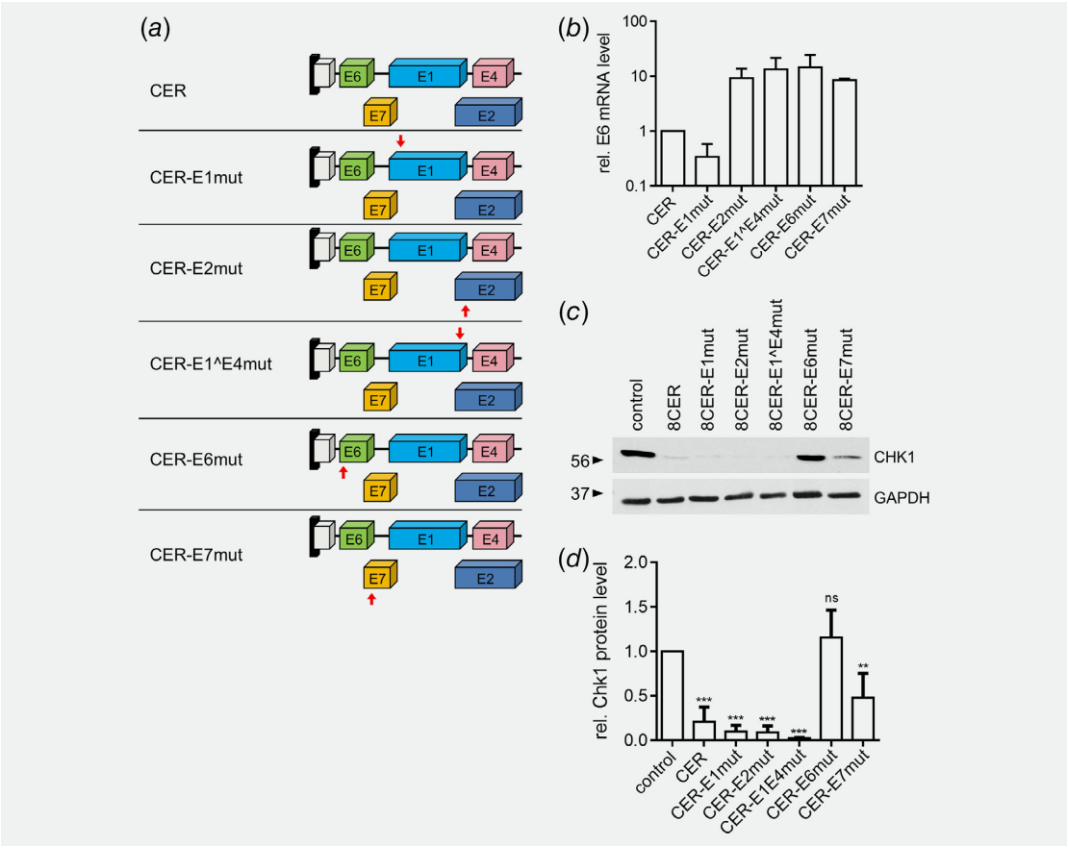

Figure 2. HPV8-E6 and -E7 are mandatory for CHK1 reduction. (*a*) Schematic diagram of the HPV8-CER genome region and the mutants originating from the pLXSN-HPV8-CER plasmid. Colored boxes: HPV8 early genes, white boxes: upstream regulatory region, arrows: inserted mutations. (*b*) Expression of E6 in PM1 cells expressing different CER mutants was confirmed by RT-qPCR (n = 2 experiments in duplicates). The oncogene expression was normalized to the housekeeping gene HPRT1. (*c*) RIPA extracts from PM1 cells expressing HPV8-CER mutants were tested for CHK1 protein expression by Western blotting. Equal loading was confirmed by immunoblotting for GAPDH. (*d*) Quantification of CHK1 protein levels detected by Western blot (n = 3) using ImageJ 1.45 s software. The values derived from the ratio of CHK1 to GAPDH are given as mean ± SD. The signal intensity in PM1 control cells was set as 1. [Color figure can be viewed at wileyonlinelibrary.com]

antibodies (Dako, Hamburg, Germany) and the BM chemiluminescence Blotting Substrate system (11500694001, Roche).

Transfection and luciferase assay and flow cytometric analysis

The generation of the luciferase reporter construct pGL2-H containing the human CHK1 promoter fragment from −2,240 to −284, as well as the empty control vector pGL2 was described elsewhere.[40] 1×10^5 cells were seeded in 6-well dishes the day before transfection. The next day, cells were transfected with the FuGene 6 reagent (Promega) in accordance with the manufacturer's recommendations. For each transfection, 3 µg of the reporter-plasmids pGL2 or pGL2-H were used. 48 h post-transfection cells were harvested, assayed for luciferase activity and normalized to the protein concentration of corresponding samples.

Flow cytometric analyses were performed to analyze 5-bromodeoxyuridine (BrdU) incorporation and total DNA content by propidium iodide (PI) treatment using the MACSQuant Analyzer (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[39]

UV irradiation of mouse lines

Mouse lines used in this study included FVB/nwt (Charles River Laboratories, Sulzfeld, Germany) and the transgenic hemizygous FVB/n line K14-HPV8-CER.[12] UV irradiation protocols were approved by the governmental animal care office North-Rhine-Westphalia (Leibnizstraße 10,45659 Recklinghausen, protocol

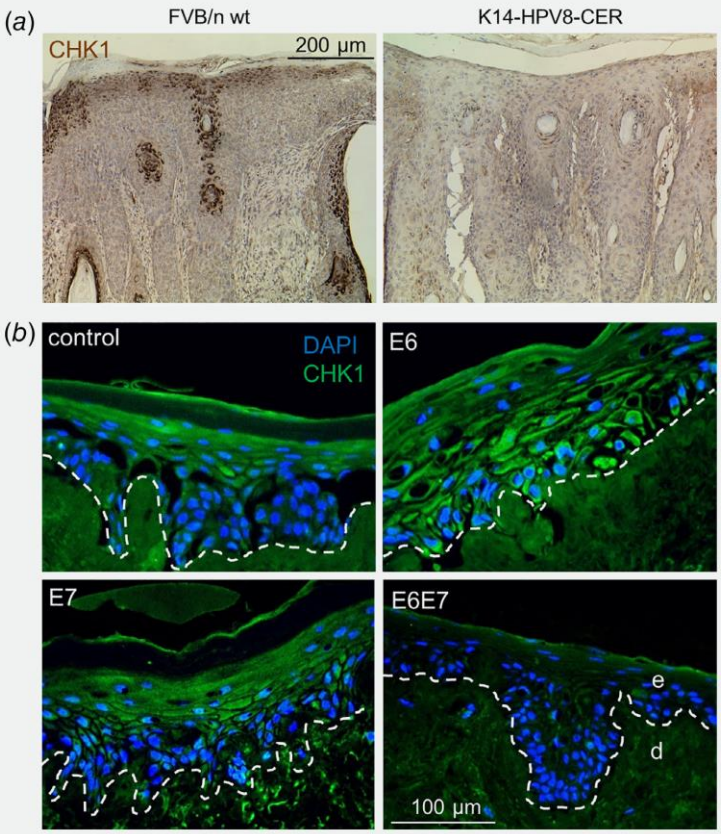

Figure 3. Stratified keratinocytes expressing E6E7 show reduced CHK1 levels. (*a*) Sections of UV-induced skin tumors from FVB/n wt (n = 1) and K14-HPV8-CER mice (n = 4), harvested 24 days after UV-treatment were immunohistochemically stained for CHK1 and counterstained with hematoxylin. (*b*) Representative immunofluorescence staining image of CHK1 on organotypic skin cultures, which were repopulated with PHK coding for the empty retroviral vector pLXSN, -E6, -E7 or -E6E7 (blue: DAPI; green: CHK1; dashed line: basement-membrane zone; d: dermis; e: epidermis). The organotypic cultures were grown for 14 days at the air-liquid interphase, followed by fixing and embedding in paraffin.

no. 8.87–50.10.35.08.163) and were in accordance with the German Animal Welfare Act as well as the German Regulation for the protection of animals used for experimental purposes. For UV treatment, age (5 weeks) and sex matched mice were shaved and irradiated once with 10 J/cm^2 UVA and 1 J/cm^2 UVB on a 4cm^2-sized dorsal caudal area. All offspring were macroscopically examined for the presence of skin lesions on day 24 after UV treatment. Directly afterwards the animals were sacrificed and pictures as well as samples of the irradiated skin were taken, fixed and subsequently embedded in paraffin.

Sections of organotypic skin cultures

The generation of organotypic skin cultures of keratinocytes expressing HPV8-E6, -E7 or -E6E7 was based on a de-epidermalized human dermis serving as the dermal equivalent, which was then repopulated with keratinocytes. These 3D cultures were grown for 14 days at the air-liquid interphase, followed by fixing and embedding in paraffin.[35,37,41]

Immunofluorescence staining of skin samples

Formalin-fixed, paraffin-embedded sections were deparaffinized in 100% xylene and rehydrated in decreasing concentrations of ethanol (100%, 90% and 70%). Antigen unmasking was performed by boiling tissue sections in 10 mM citric buffer for 3 min followed by 15 min resting at room temperature (RT). Blocking of unspecific antigen sites was achieved with 50% goat serum in PBS for 1 h at RT. Organotypic skin sections were co-stained overnight at 4°C with antibodies targeting CHK1 and microtubule-associated protein light chain 3B (LC3B, 2775, Cell Signaling, Frankfurt, Germany). Following extensive washing, the sections were incubated with anti-mouse secondary antibodies conjugated with Alexa Fluor 488 (A-11029, Life

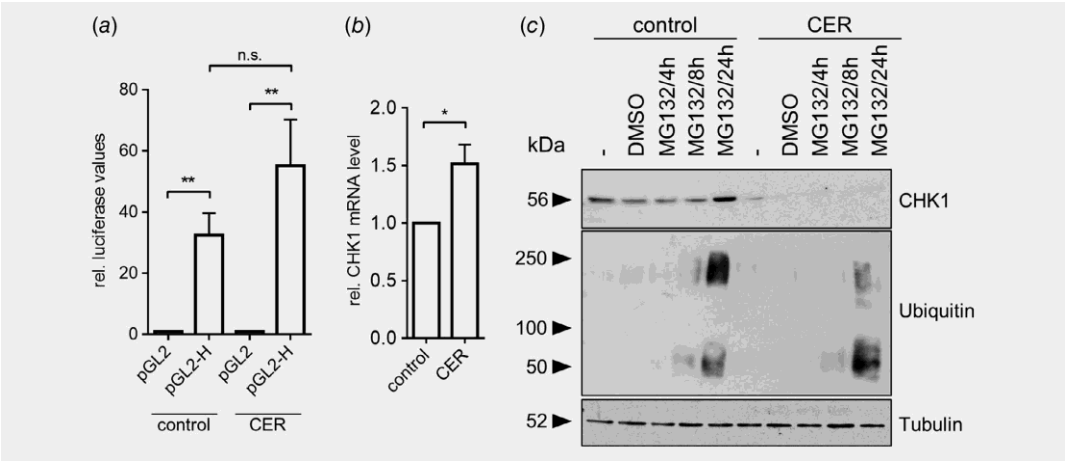

Figure 4. HPV8-CER does not target CHK1 at the transcriptional level or through proteasomal degradation. (*a*) RTS3b cells were transiently co-transfected with pLXSN or pLXSN-CER together with the luciferase reporter construct (pGL2-H) containing the fragment −2,240 to −284 of the human CHK1 promoter in front of the luciferase gene or the empty luciferase vector (pGL2). 48 h post transfection, luciferase activity was measured and normalized to protein concentration. The bars represent the mean ± SD of duplicate measurements from $n = 3$ independent experiments. The relative luciferase value of the corresponding control was set as one. (*b*) CHK1 mRNA expression was quantified by RT-qPCR in PM1-control and PM1-HPV8-CER. The bars represent the mean ± SD of duplicate measurements from $n = 3$ independent experiments. CHK1 mRNA levels were normalized to GAPDH mRNA levels. The relative gene expression level in PM1-pLXSN was set as one. (*c*) Representative immunoblot of CHK1 protein expression and mono- and polyubiquitinylated conjugates in PM1-control and PM1-HPV8-CER cells treated with DMSO or 50 μM MG132 for for 4, 8, and 24 h ($n = 3$). Equal loading was confirmed by immunoblotting for tubulin.

Technologies, Carlsbad, CA) and anti-rabbit secondary antibodies conjugated with Alexa Fluor 594 (A-11012, Life Technologies) for 1 h at RT. After counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), fluorescence images of the slides were acquired with a Leica DMI 6000B microscope equipped with a Leica DFC365 FX camera and analyzed with Leica LAS X imaging software (v3.3.0.16799).

Statistical analysis

Promoter assays and RT-qPCRs were repeated at least three times in duplicate, and the results are presented as mean ± SD. Statistical significance was determined with an unpaired 2-tailed Student's t-test. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control conditions (n.s., not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). Images from immunoblots or immunohistochemical stainings are from a representative experiment and were qualitatively similar in $n = 3$ experiments.

Results

CHK1 is absent in keratinocytes simultaneously expressing HPV8 E6 and E7

To analyze whether HPV8 early genes may target CHK1 protein levels in skin cells, PHK were first transduced with either recombinant retroviruses encoding for HPV8 proteins E2, E6, E7 individually or HPV8-CER.[11,35] In comparison to cells expressing the empty retroviral vector pLXSN, keratinocytes expressing HPV8 E2, E6 or E7 alone showed no effect on CHK1 protein levels. Most intriguingly though, a complete absence of CHK1 protein was observed in cells expressing HPV8-CER (Fig. 1*a*). mRNA expression of the individual viral genes in the different cell lines is shown in Figure 1*b*. FACS data suggest that the absence of CHK1 in CER cells might contribute to the observed elevated percentage of cells passing through the S-phase ($p = 0.037$) (Fig. 1*c*). In order to identify the viral gene responsible for CHK1 downregulation, we further assessed CHK1 protein levels in E1^E4 as well as E6E7 expressing cells. As shown in Figure 1*d* CHK1 is not a target of E1^E4. However, it is clearly absent in cells expressing E6E7 (Fig. 1*e*).

To further clarify the effects of individual HPV8 early viral proteins on CHK1 expression levels, stop codons were introduced into the pLXSN-8CER construct to specifically delete individual viral genes (Fig. 2*a*). Viral gene expression from the mutated CER constructs was verified through RT-qPCR for E6 (Fig. 2*b*). Deletion of E1, E2, or E1^E4 had no discernible effect on CHK1 protein levels. In contrast, silencing E6 or E7 within the CER rescued CHK1, implicating that both oncogenes contribute to CHK1 abrogation, with E6 being the dominant oncogene under these experimental conditions. (Figs. 2*c* and 2*d*).

To demonstrate that HPV8 targets CHK1 also in differentiating epithelial cell layers, we analyzed CHK1 protein levels *in vivo*. To this end, UV-induced skin tumors of K14-HPV8-CER mice ($n = 4$) as well as a UV induced skin tumor of a FVB/n wt mouse, all taken 24 days post treatment, were

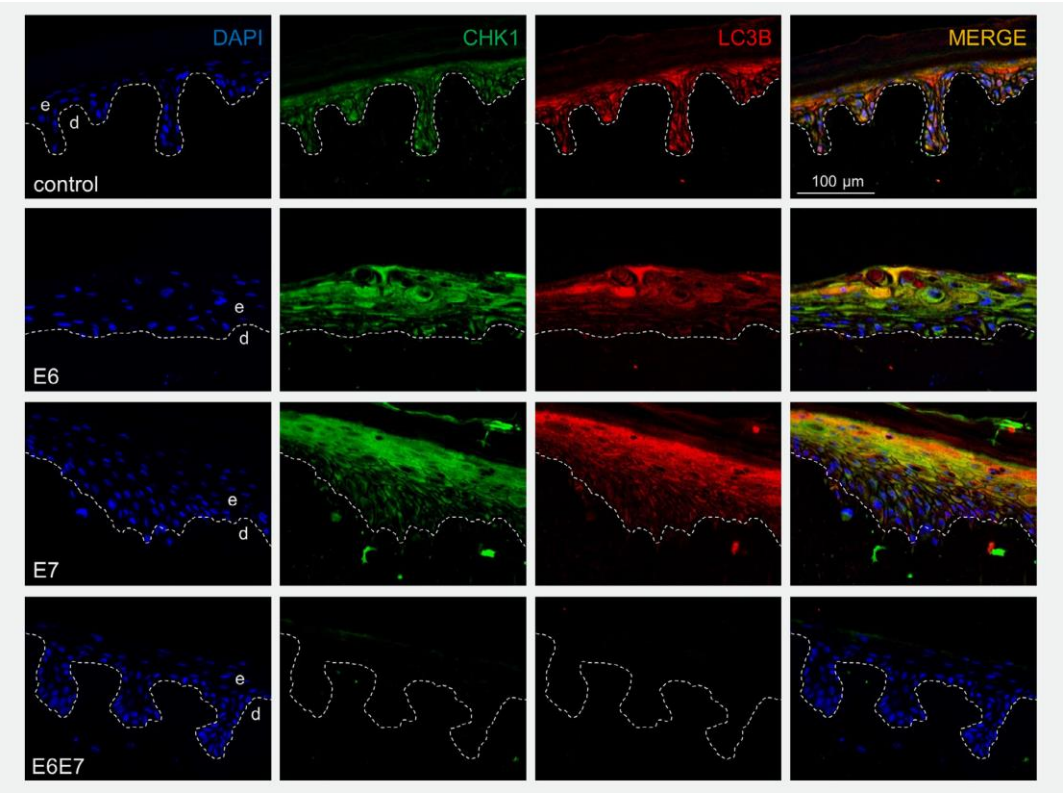

Figure 5. CHK1 and LC3B expression is only abrogated in E6E7 expressing keratinocytes differentiated in organotypic skin cultures. Representative immunofluorescence staining images of CHK1 and LC3B on organotypic skin cultures, which were repopulated with PHK coding for the empty retroviral vector pLXSN, –E6, –E7 or -E6E7 (blue: DAPI; green: CHK1; red: LC3B; dashed line: basement-membrane zone; d: dermis; e: epidermis; $n = 3$). The cultures were grown for 14 days at the air-liquid interphase, followed by fixing and embedding in paraffin.

immunohistochemically stained. The UV-induced skin tumors from the K14-HPV8-CER mice clearly showed the absence of CHK1 compared to the tumor of the irradiated FVB/n wt mouse (Fig. 3*a*). In addition, we also stained E6, E7 or E6E7 expressing organotypic skin cultures for CHK1. These results showed that—whereas CHK1 is expressed in E6 or E7 positive cultures—expression is completely abrogated in E6E7 cultures (Fig. 3*b*). To conclude, our results strongly imply that both E6 and E7 are indispensable for CHK1 dysregulation.

Regulation of CHK1 is not affected by transcriptional repression or proteasomal degradation

To address the question whether CHK1 dysregulation by HPV8 might be achieved through promoter repression, we performed transient reporter gene assays by transfecting RTS3b cells with pLXSN (control) or pLXSN-HPV8-CER with the luciferase construct pGL2-H, comprising the human CHK1 promoter from −2,240 to −284.[40] In pLXSN transfected cells the CHK1-promoter construct showed an about 30-fold increase in luciferase activity compared to the empty luciferase vector pGL2 ($p = 0.0016$). HPV8-CER activated the promoter construct pGL2-H about 55-fold ($p = 0.0034$), with luciferase activity not significantly differing from levels observed in pLXSN transfected cells. The difference in CHK1 promoter activity was not statistically significant ($p = 0.0783$) when comparing the activity in pLXSN and HPV8-CER transfected cells (Fig. 4*a*). These results suggest that HPV8 does not target CHK1 expression *via* promoter deregulation. This hypothesis was further underpinned by RT-qPCR results, showing an only 1.5-fold increase of CHK1 mRNA levels in PM1-HPV8-CER cells compared to PM1-pLXSN (Fig. 4*b*).

We next investigated whether HPV8-CER may affect CHK1 levels through ubiquitin-mediated proteasomal degradation. To block proteasomal activity, PM1-pLXSN and PM1-HPV8-CER cells were treated with the proteasome inhibitor MG132. Curiously, while CHK1 levels in PM1-pLXSN showed a slight increase 24 h post treatment, this did not result in an elevation of CHK1 protein levels in PM1-HPV8-CER cells.

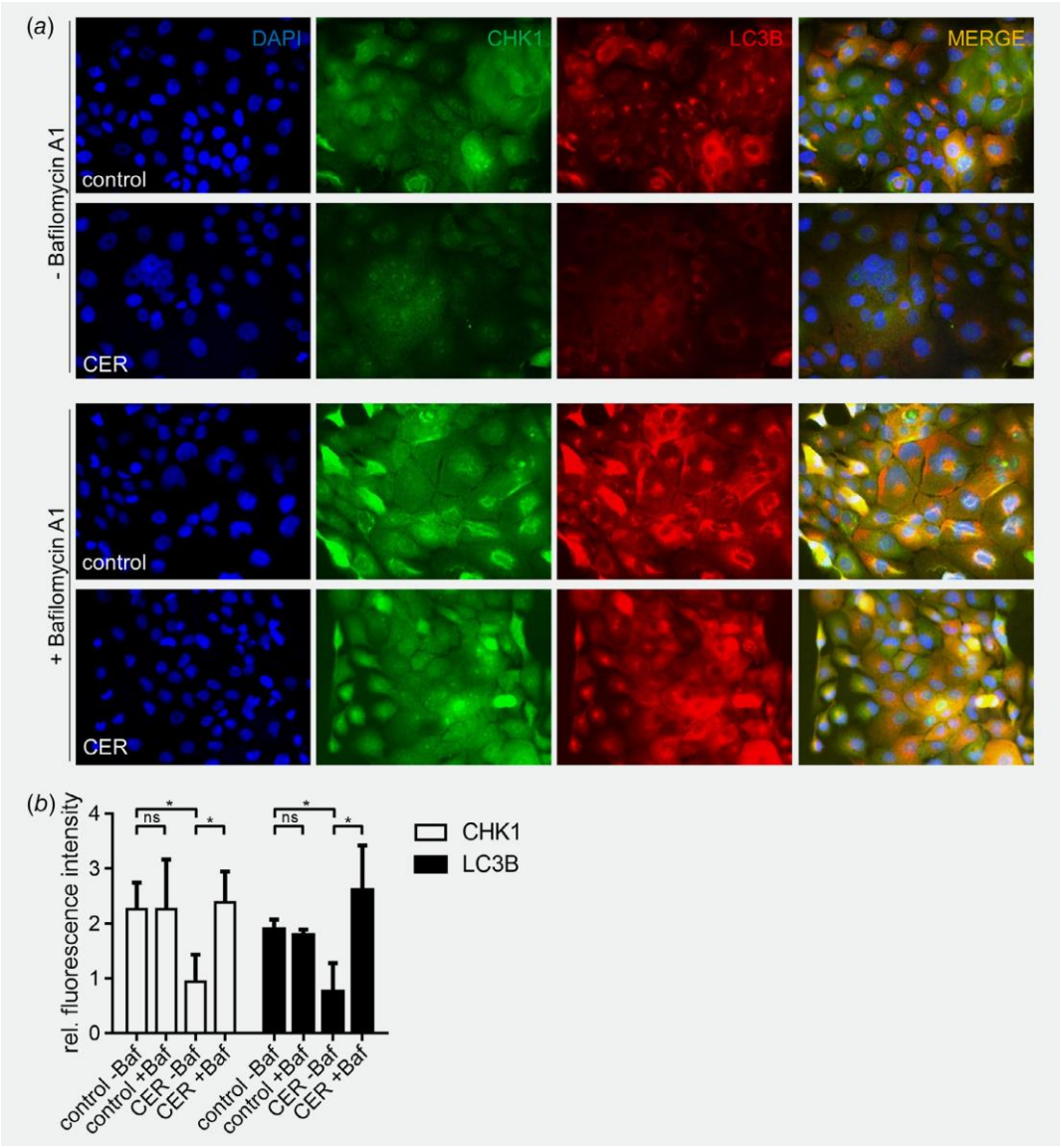

Figure 6. Inhibition of autophagic processes leads to an accumulation of CHK1 in keratinocytes expressing HPV8-CER. (*a*) Representative immunofluorescence staining images of CHK1 and LC3B in control and CER-expressing keratinocytes left either untreated or subjected to the autophagy inhibitor Bafilomycin A1 (blue: DAPI; green: CHK1; red: LC3B). (*b*) Quantification of CHK1 and LC3B fluorescence signals ($n = 3$) using ImageJ 1.45s software. The values derived from the ratio of CHK1 or LC3B to DAPI are given as mean ± SD.

The increase of total levels of ubiquitinated proteins after MG132 treatment in both cell lines verified efficient blocking of the ubiquitin-proteasome by MG132, which proved that HPV8-CER expression does not result in CHK1 proteasomal degradation (Fig. 4*c*).

HPV8 dysregulates CHK1 by influencing autophagic processes

To investigate a possible involvement of autophagic processes in CHK1 regulation, organotypic cultures were co-stained for CHK1 and LC3B, a subunit of the autophagosome. Thus,

immunofluorescence staining of LC3B has become a reliable method for monitoring autophagy and autophagy-related processes in the skin.[42,43] Figure 5 demonstrates, that in control as well as E6 or E7 expressing organotypic cultures, CHK1 is expressed more strongly in suprabasal keratinocytes than in basal cell layers, with a more cytoplasmic rather than nuclear staining pattern. The CHK1 distribution pattern was also paralleled by LC3B expression. In contrast, the complete absence of CHK1 and LC3B in the E6E7 culture strongly supports the notion of a link between active autophagic processes and CHK1 protein expression.

To finally demonstrate that CHK1 is autophagy-dependent, control and HPV8-CER expressing keratinocytes were left either untreated or treated with the autophagy inhibitor Bafilomycin A1. As expected, in untreated cells, CHK1 levels were reduced in HPV8-CER expressing cells when compared to the control. This decrease was paralleled by low LC3B levels. Inhibition of autophagy with Bafilomycin A1 did neither affect CHK1 nor LC3B levels in control cells. Interestingly, in HPV8-CER cells treatment led to an accumulation of LC3B as well as CHK1 (Figs. 6*a* and 6*b*), indicating that HPV8 mediates CHK1 repression through activation of autophagic processes.

Discussion

In this study, we demonstrate for the first time a reduction of total CHK1 protein levels in HPV8 positive keratinocytes. In a previous study we had already shown that following UV irradiation, CHK1 phosphorylation was inhibited by HPV8-E6, whereas unexpectedly, total CHK1 levels remained unaffected.[16] Since the influence of HPV8-CER on CHK1 had not been addressed in our former study, we tested the effect of simultaneous HPV8 protein expression on CHK1 regulation. Interestingly, we found that HPV8 is able to lower CHK1 total levels, but this was only the case when both E6 and E7 were simultaneously expressed. Therefore, we conclude that both viral proteins are required for CHK1 repression.

As analyses of HPV oncogenic functions are linked to the differentiation status of infected epithelia, data from *in vitro* studies may lead to mis-interpretations regarding potential central roles *in vivo*. Therefore, we further tested, and thus proved our *in vitro* observations *in vivo* in skin tumors originating from K14-HPV8-CER transgenic mice and 3D skin models of HPV8-E6E7 expressing human keratinocytes. Our *in vivo* results suggest a mechanism, by which HPV8 oncoproteins may utilize the autophagic pathway to manipulate CHK1 levels. In addition, Hong and colleagues recently discovered that CHK1 is targeted by the high-risk alphaHPV types HPV16 and HPV31 through upregulation of the microRNA miR-424.[44] We now demonstrate that betaHPV also target total levels of CHK1, yet possibly by another mechanism.

As CHK1 promoter activity was not affected by HPV8-CER expression, we could exclude a transcriptional involvement, that—in principle—could have been deregulated by HPV8 early proteins.[41,45] Many cellular proteins are known to be targeted for degradation by HPV-E6 utilizing the ubiquitin proteasome pathway.[46,47] Here, we now show that this pathway is not the governing mechanism controlling CHK1 protein levels. Intriguingly, we identified CHK1 expression to be paralleled by expression of the autophagy marker LC3B. Moreover, when repressing the autophagic flux *in vitro* with Bafilomycin A1, CHK1 levels were rescued and an accumulation of LC3B was observed, implicating that autophagy is targeted by HPV8. However, to get a more comprehensive understanding regarding the mechanism in HPV infected human skin, this would need to be addressed in more detail in a follow up study.

In normal human skin, autophagy is a physiological process which plays a critical role in normal epidermal development and differentiation. Furthermore, autophagy provides protection from UV-induced damage as a pro-survival mechanism.[42,48] It is generally accepted that a critical step in the pathogenesis of betaHPV is the prevention of host cell death following UV exposure.[16,19] Therefore, the absence of CHK1 in infected skin may allow virus-infected cells to persist in a replicative state. This hypothesis is supported by our own observations, as PM1-HPV8-CER cells have a higher percentage of cells in the S-phase of the cell cycle. In line with this idea, other studies have shown that CHK1 inhibition can lead to an increase in DNA replication, yet also an increase in DNA damage.[49] Furthermore, it is interesting to note that autophagy deficiency impairs UV-induced DNA damage repair mechanisms involving the cellular regulatory protein p300.[50] Inhibition of CHK1, as well as autophagic processes, may therefore predispose cells for accumulation of UV-induced DNA damage.

In summary, our study unravels the pivotal role of autophagy as a regulatory mechanism, hijacked by HPV8 to control CHK1 protein levels, which may play a significant role in viral life cycle, epithelial homeostasis and even most importantly, carcinogenic processes.

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IV. Discussion

In works preceding this thesis our group had already shown that the betaHPV oncoprotein E7, which is the main viral protein involved in reprogramming skin keratinocytes show a drastically increased tendency to migrate and invade underlying dermal compartments. The basis of my work was the strong implication that the fibronectin α3β1 axis was the major causative signaling for this process to (Heuser et al., 2016b). The major goal of this thesis therefore was to arrive at a deeper understanding of how E7 hijacks the host cell transcriptome, proteome and metabolism to cause this transformed keratinocyte phenotype.

Publication no. 1: (Kirschberg et al., 2019)

To shed more light on how HPV8-E7 may interfere with the host cell transcriptome we used cDNA microarray data generated with RNA extracted from HPV8-E7 keratinocytes. We also benefitted from the fact that cooperation partners at the DKFZ in Heidelberg had also generated microarray data for HPV8-E7 positive cells which we compared with our own data sets. This turned out to aid us greatly in finding differentially expressed genes in the biological replicates. Firstly, we identified that the global effect of HPV8-E7 on cellular transcription is mainly mediated through Sp1/3 transcription factor binding sites within the promoters of cellular genes. Although the transcription factor family is comprised of four known members, namely Sp1, Sp2, Sp3 and Sp4 there is only a very marginal overlap regarding their function amongst the different Sp-family members. There is only one exception, and that is the interaction of Sp1 and Sp3, which play a dual role regarding regulation of gene expression through very similar and thus overlapping Sp1/Sp3 binding sites (Huang et al., 2015). Both Sp1 and Sp3 together exert control over more than 12.000 genes, and the two family members are therefore very often referred to as simply Sp1/3 (Li and Davie, 2010). It is highly dependent on the binding region within a given promoter or Sp1/3 relative ratios whether Sp3 may act as an activator or repressor of gene expression. The same is true for Sp1. Furthermore, there is increasing evidence that deregulation of Sp transcription factors plays a pivotal role in tumorigenesis and adds to the metastatic potential of many tumor types (Mertens-Talcott et al., 2007). The effect in HPV8-E7 positive cells seems to be mainly the result of Sp3 overexpression with no obvious Sp1 deregulation.

However, we have not analyzed whether E7 actually binds to the Sp1/3 complex. Interestingly, Sp3 overexpression seems also to be key in upregulating fibronectin expression in HPV8 infected keratinocytes. When the Sp1/3 binding site was mutated within the promoter region of the fibronectin gene the effect of E7 was diminished demonstrating that there seemingly is a transcriptional de-regulation of fibronectin expression by E7 and subsequent testing whether HPV8-E7-mediated activation of FN still took place or not, we could prove that HPV8-E7 controls the FN promoter activation through a specific Sp1/3 binding motif. Interestingly the observation that the HPV8-E7^{L23A} invasion-deficient mutant was also far less proficient at inducing Sp3 and FN expression further underpins that the E7 protein directly influences Sp3 and may be involved in controlling FN dependent processes regulating keratinocyte invasion. In addition, and most unexpectedly Sp3 and fibronectin are not only overexpressed in E7 positive keratinocytes *in vitro*, but also show a strong nuclear localization pattern in skin tumors of EV patients. (Kirschberg et al., 2019). It was reported decades ago that cellular FN apparently can be part of the nuclear matrix of cancer cells. In hepatocellular carcinoma cells FN was even found in the nuclear matrix of cells cultivated in FN deprived medium and that it was even preferentially associated with the nuclear matrix (Jagirdar et al., 1985). Interestingly, such nuclear deposition has also been described in HeLa cells (Zerlauth et al., 1988).

In cervical carcinomas FN staining patterns were described as diffuse (Goldberg et al., 1998), which does not exclude the possibility that nuclear FN may indeed also be found in these cancers (Zerlauth et al., 1988). Regarding the modulation of FN expression by other HPV it has previously been described that both the HPV16-E6 and -E7 proteins are able to promote FN expression in monolayer cultures of primary keratinocytes (Hellner et al., 2009). While it has been known that extracellular FN regulates keratinocyte homeostasis, it is completely unknown whether the presence of nuclear FN may be a result of alterations to the FN molecule – for instance by changing binding affinities – or if it is a consequence of changes of the tumor nuclear matrix. It is also entirely unclear if nuclear FN expression has any influence skin on cell motile behavior or if the observed deposition in the nuclear matrix serves other unknown purposes. Furthermore, very little research has been done and the existence of FN being present at the nucleus has not been further researched in decades. The last publication I could find about nuclear fibronectin after an extensive pubmed search goes back to 1988 (Zerlauth et al., 1988).

The described results therefore most certainly warrant further studies to underscore a potential clinical importance of FN deregulation in betaHPV-mediated skin carcinogenesis. Furthermore, very little research has been done and the existence.

Publication no. 2: (Kirschberg et al., 2021)

In addition to analyzing upstream regulators involved in E7-mediated deregulation of cellular gene expression we then went on and analyzed the downstream effects of E7. Here, we identified GADD34 (growth arrest and DNA damage-inducible protein 34), also known as PPP1R15A (protein phosphatase 1 regulatory subunit 15A) and GDF15 (growth/differentiation factor 15) as novel and strongly activated genes in HPV8-E7 infected keratinocytes. GADD34 is a family member of genes induced by DNA damage and apoptotic processes and coordinates these mechanisms in response to DNA damage (Grishin et al., 2001). GADD34 is known to be able to dephosphorylate a variety of kinases that play a role in important signaling cascades. It is also involved in recruiting a catalytic subunit from one of various protein phosphatase I (PP1) isoforms which leads to the formation of a holophosphatase complex that has the ability to dephosphorylate several enzymes *in vitro* (Harding et al., 2009). Furthermore, it has recently been demonstrated that increased GADD34 levels result in a dephosphorylation of eIF2a that is catalyzed by the GADD34/PP1 complex. In contrast, phosphorylated eIF2a (eIF2a-P) exerts control over GADD34 expression, which is consistent with the fact that this feedback-loop targeted by E7 may be of paramount importance in controlling keratinocyte differentiation (Collier et al., 2017). This observation may point towards a role of this regulatory axis in manipulating the growth of undifferentiated basal keratinocytes and therefore warrants further research.

GDF15, originally identified as MIC-1, is a member of the TGF-β superfamily. However, its role in keratinocyte biology has thus far not been extensively studied. It is known that GDF15 overexpression in melanoma cells is associated with tumor invasion and metastasis (Unal et al., 2015). In line with our data, it is reasonable to speculate that the E7-mediated upregulation and secretion of GDF15 may disturb the physiological keratinocyte differentiation program and epidermal-dermal crosstalk, that may contribute to aberrant cell growth. However, the exact role of GDF15 in tumorigenesis and progression is not really understood. Some literature suggests that the protein acts as a tumor suppressor, while others proclaim that it drives the development of cancers.

These seemingly opposing effects of GDF15 in the context of tumor biology may, however, also be dependent on the tumor stage and possibly also the cancer location. For instance, it is known that GDF15 is strongly expressed in a vast number of tumors such as breast, pancreas, lung, prostate cancer as well as melanoma (Brown et al., 2003;Kadara et al., 2006;Baek et al., 2009;Brown et al., 2009;Youns et al., 2011). GDF15 binds to the ECM of prostate cancer cells and is one of the most viable parameters to assess tumor progression (Bauskin et al., 2005). Furthermore, the protein is not only known to play a role in carcinogenesis, but it also exerts control over cellular stress response pathways (Unal et al., 2015). How GADD34 and GDF15 are transcriptionally controlled by E7, possibly in conjunction with a potential involvement of Sp1/Sp3, or if E7 interferes with phosphorylation and thereby activation of eIF2a (which is known to regulate GADD34 expression) would warrant further investigation. The observation that GADD34 and GDF15 are highly upregulated in only undifferentiated HPV8-E7 expressing keratinocytes but that this effect is significantly reduced in differentiating keratinocytes may point towards a more important role of gene expression changes in undifferentiated basal keratinocytes which are coincidentally also the keratinocytes which primarily display invasive behavior. Also, since these experiments were performed with primary keratinocytes grown only on plastic follow-up experiments showing the effect of different ECM proteins including FN should be used in follow-up studies. Also, testing of clinically approved GADD34 and GDF15 inhibitors in our K14-HPV8 transgenic mouse models and later in clinical studies would greatly contribute to expand our understanding of their potential role in the context of betaHPV-associated carcinogenic potential. Another regulatory level which I discovered was that E7 is not only able to modulate the transcriptome but also has an effect on the proteome / phosphoproteome. It is known that the E7 protein itself is a phosphorylation target of the casein kinase II (Iftner et al., 1990). Quite surprisingly, while proteomic data have indeed been generated for HPV16-E7, phospho-proteomic data for the E7 protein have not been published to the best of my knowledge. In our analyses of the phosphoproteome I did not only include total cell extracts from wild type E7 expressing keratinocytes grown on fibronectin, but also cells expressing the invasion deficient mutant $E7^{L23A}$ to increase the likelihood to identify proteins that may most likely be involved in keratinocyte invasion. Our data generated from the phospho-proteomics and proteome analyses strongly implicate that particularly the scaffold protein 14-3-3 and the kinases CKII as well as ERK are involved in phosphorylation

deregulation. Interestingly, also the Src-kinase family members Lyn and Fyn seem to play a pivotal role in HPV8-E7-mediated keratinocyte transformation. These kinases seem to specifically hyperphosphorylate cellular proteins that are involved in cytoskeletal organization and cell polarity, among others. Curiously, identified hypophosphorylated proteins are mainly grouped in DNA replication and DNA damage repair pathways. Considering that CKII is already very well described and known to be vital for normal E7 function (Basukala et al., 2019;Piirsoo et al., 2019) it was not studied in-depth. This kinase is an anti-apoptotic kinase which is known to be upregulated in a variety of cancers (reviewed in (Franchin et al., 2017). 14-3-3 and ERK are also known to be important signaling kinases for HPV induced tumors (Sichtig et al., 2007;Luna et al., 2021).

The Src family cytoplasmic tyrosine kinases (SFK) are important for signal transduction and are activated by a variety of cell-surface receptors including receptor-tyrosine kinases, integrins or extracellular matrix receptors. The family is comprised of 11 homologous members, namely Src, Fyn, Yes, Blk, Yrk, Frk, Fgr, Hck, Lck, Srm, and Lyn. Src, Fyn and Yes seem to be ubiquitously expressed throughout different tissue types including keratinocytes of squamous epithelia. They may also exhibit tumorigenic properties when their activity is upregulated as it is often observed in solid tumors and such deregulation is typically associated with advanced tumor grades and metastasizing of the primary tumor (summarized in (Szalmas et al., 2013))**.** Intriguingly our analyses revealed that only Lyn and Fyn seem to become activated in HPV8-E7 positive keratinocytes grown on fibronectin. In this regard, Lyn has also been implicated to be upregulated in response to DNA damage and is the only kinase that is activated following genotoxic stress, which is associated with cell cycle arrest and apoptosis (Yoshida et al., 2000). Furthermore, Lyn acts as a negative regulator of GADD34 in the context of DNA damage-induced cell death (Grishin et al., 2001). On the other hand, Fyn uses alternative splicing to adapt to different cellular environments. There are three known variants of Fyn, namely FynT, FynB, and FynC, which arise from alternative splicing of exon 7 of the Fyn gene, which has an effect on auto-inhibition of Fyn (Brignatz et al., 2009;Uddin et al., 2020). The observation of a fourth band implicates that an additional Fyn isoform exists in HPV8-E7 positive cells grown on fibronectin. This could mean that we may have discovered a novel Fyn variant, which would then likely be associated with E7 expression and thus might be relevant for changes in the microenvironment of betaHPV-positive skin.

Interestingly, in the past a phosphokinase array was carried out for HPV16-E7. Here, the authors described that E7 leads to phosphorylation of the ubiquitously expressed Src family kinases Src, Yes and Fyn. Curiously, the regulation of these kinases only took place on the protein level as mRNA levels remained unchanged.

Publication no. 3: (Oswald et al., 2019)

A third regulatory level I studied in more detail were direct protein-protein interactions studies to identify HPV8-E7 cellular binding partners. Here, we identified NuMa (nuclear mitotic apparatus protein 1) as a previously unknown interaction partner of HPV5-E7 and HPV8-E7. NuMa is a protein involved in the establishment of cell polarity in the skin, a prerequisite for asymmetric cell division, required for normal differentiation of healthy skin. The basal cell layer also encompasses the epidermal stem cells, which regularly undergo asymmetric cell divisions where one daughter cell retains the basal, undifferentiated properties, whereas the other cell commits itself to differentiation. NuMa is a key component located at the nuclear matrix and of vital importance in the re-assembly of the nucleus once mitosis has been completed (Sun and Schatten, 2006). During mitosis NuMa tightly controls proper organization of microtubules at centrosomes and correct chromosome alignment as the cells prepares for cell division. Known NuMa functions are known to be involved in spindle microtubule organization, which is regulated by RanGTP and Pins-related protein LGN (also called G-protein-signaling modulator 2) (Lechler and Fuchs, 2005). NuMa is also required, together with dynein, for the positioning of minus ends of the microtubules at the poles of the mitotic spindle during mitosis (di Pietro et al., 2016). The orientation of the mitotic spindle is vital for morphogenesis, asymmetric cell division and thus also maintenance of the stem cell population in skin tissue. There is rising evidence that interference with normal spindle orientation is involved in a number of malignant processes including carcinogenesis. It was previously shown in HeLa cells that the spindles are aligned in accordance to the cell-ECM adhesion plane, which serves as a safety net to ensure that both daughter cells will remain connected to the ECM following symmetric mitosis (Toyoshima and Nishida, 2007). Interestingly, correct spindle adjustment is also mediated by integrin cell-substrate adherence. Apart from dynein, the LGN protein is a key player as far as correct spindle orientation is concerned. LGN and a phosphorylation of NuMa at the tyrosine residue 1774 govern correct spindle organization.

The LGN/NuMa/dynein-dynactin complex is highly conserved and responsible for correct spindle orientation in various cell types including epidermal cells (Seldin et al., 2016) (described in detailed in (Matsumura et al., 2012)). NuMa knockdown experiments resulted in misaligned centrosomes, which demonstrates that correct spindle orientation requires a functional binding of NuMa to LGN (Williams et al., 2011;Seldin et al., 2016). In healthy adults, stratification homeostasis seems to build upon symmetric and asymmetric (90° angle spindle orientation) cell divisions. One characteristic of these mitotic spindle orientations is that one daughter cell retains contact with the basement membrane and also retains stemness, whilst the other daughter cell loses it and begins to differentiate whilst moving upwards through the epidermal layers. It has been known for quite some time that a vast number of key functions such as ECM secretion or integrin-associated focal adhesion are localized in the basal layer, and these 90° cell divisions represent a natural tool for the unequal division of the two daughter cells during the mitosis of stem cells, which also means that such perpendicular cell divisions govern not only proper stratification of the epidermis but also asymmetric cell divisions of basal stem cells (Schlaepfer and Hunter, 1996;Mariotti et al., 2001;Fuchs and Raghavan, 2002;Lechler and Fuchs, 2005). When a cell in the basal cell layer undergoes an asymmetric division there is a more profound presence of NuMa at the apical surface of such basal cells. Interestingly, in basal keratinocytes undergoing mitosis dynactin is found in the same location as NuMa, suggesting that pulling forces of NuMa, dynein and dynactin at the apical side of the cells contribute to these processes (Lechler and Fuchs, 2005).

In our study (Oswald et al., 2019) we made the most intriguing observation that a small fraction of cells in monolayer cultures showed a co-localization of E7 with NuMa at only one of the spindle poles during mitosis. In addition, in OSCs of HPV8-E7 expressing keratinocytes we mainly found an increase of NuMa expression in suprabasal cells rather than a profound change in its distribution. Quite curiously and unexpectedly, analyses of SCCs taken from different EV patients showed peculiar NuMa staining patterns. While we could detect nuclear localization - as expected - we also found cytoplasmic localization of NuMa in some tumors. A possible shift in the balance of asymmetric / symmetric cell division towards symmetric cell division by E7 binding to NuMa in the basal cell layer – the primary site of virus infection – might elevate the number of stem-like cells and could possibly aid neoplastic developments. What we already know is, when expressed in primary human keratinocytes, E7 does have an

effect on the stem cell pool and increases the number of stem cell-like cells, as demonstrated in colony-formation and tumor sphere assays previously carried out in our group (Hufbauer et al., 2013). Asymmetric cell division is a prerequisite for epithelial differentiation, with the latter of which also being tied to the completion of the HPV replication life cycle, which is strongly coupled with the differentiation status of cells within the epithelium (Nguyen and Munger, 2009;Hasche et al., 2018).

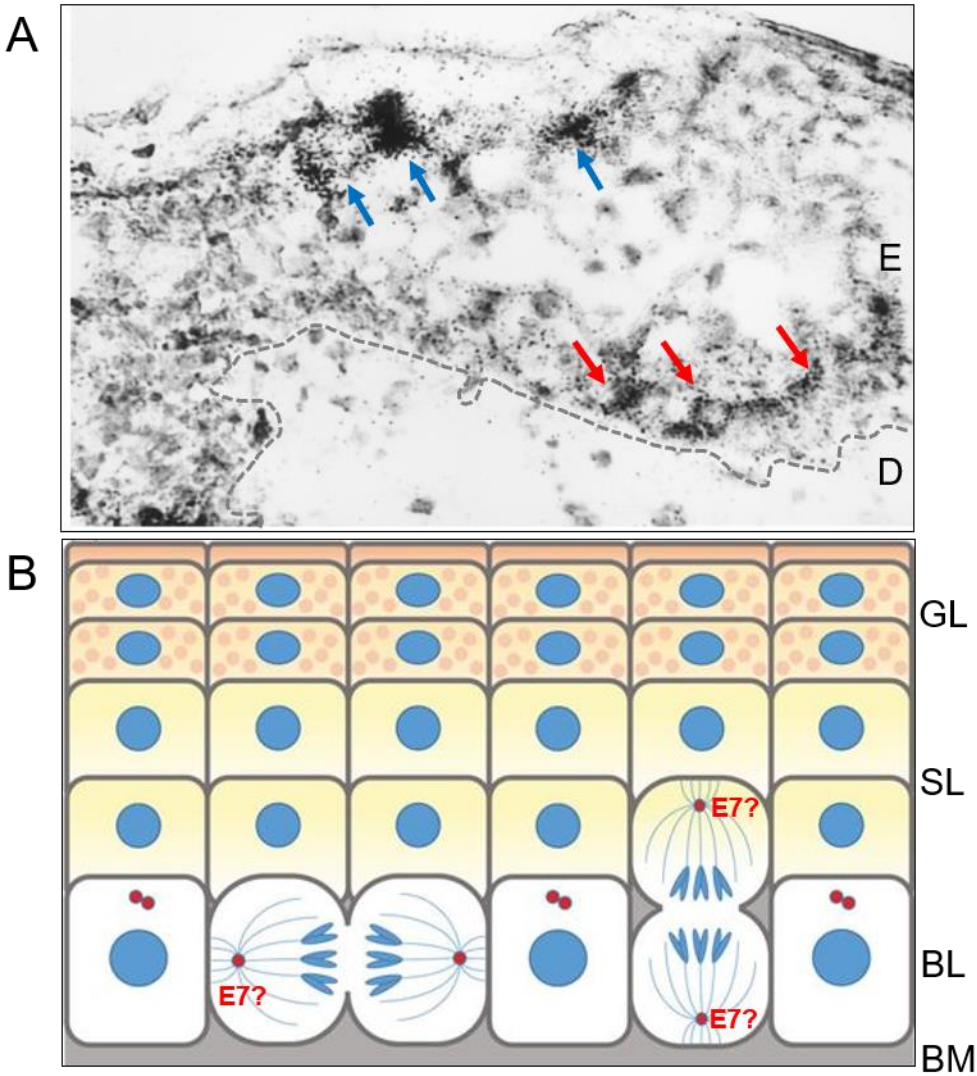

Fig. 6 Possible effect of E7 / NuMa co-localization on symmetric or asymmetric cell divisions in the epidermis. (A) In situ-hybridization against E7 transcripts in a HPV5 positive benign lesion of an EV patient was performed by Haller et al., 1995 to investigate the cell differentiation-dependent expression of viral transcripts. E7-specific in-situ signals were found in both basal (red arrows) as well as suprabasal cell layers (blue arrows) (D: dermis; E: epidermis) (adapted and modified from (Haller et al., 1995)). (B)The epidermis is a multi-layered structure whose inner most layer, the basal layer, contains progenitor cells that can orient their mitotic spindle parallel or perpendicular to the underlying basement membrane to undergo symmetric (cell division on the left) or asymmetric division (cell division on the right) respectively. The latter divisions result in a basal progenitor cell and a differentiating cell positioned in the suprabasal layer. A possible role of E7 at either basal or apical dividing cells might have an impact on proliferation and differentiation processes in betaHPV infected skin and may also have an effect on the stem cell pool (BM: basement membrane; BL: basal cell layer; SL: squamous cell layer; GL: granular cell layer) (adapted and modified from (Kulukian and Fuchs, 2013)).

However, since the expression of E7 is not only active in basal keratinocytes, but it is also expressed in more suprabasal layers (Haller et al., 1995), these expression patterns are most likely vital for normal completion of the viral life cycle. It would be interesting to study in more detail the ratios of asymmetric cell division to clarify on which spindle pole (apical / basal) during asymmetric cell division E7 is predominantly located (Fig. 6). It is therefore of high significance to arrive at a deeper understanding of the molecular basis which orchestrates NuMa distribution and to understand on which spindle pole (apical / basal) E7 is predominantly located during asymmetric cell division ratios (Fig. 6) in betaHPV-infected epithelial tissues. It would also warrant further research to understand whether FN – since we know that ECM signaling is known to affect NuMa distribution and additionally plays a role in cell differentiation and carcinogenic processes (Lelievre et al., 1998) – may also contribute to the carcinogenic potential of E7. Such an effect has so far not been described for potential betaHPV E7 / NuMa regulatory circuits which may be exerting control over the stem cell pool. Such observations have, to the best of our knowledge, only been reported for high-risk alphaHPV thus far (Nguyen and Munger, 2009). The molecular mechanisms for the correct or possibly disturbed orientation of the mitotic spindle in HPV infected tissues still requires detailed research that needs to be conducted to fully understand the process.

Publication no. 4: (Kirschberg et al., 2020)

It remains an unresolved mystery how HPV manages to gain control over the host cell energy metabolism to meet the heightened energy requirements. Previously it was described that basically all high-risk alphaHPV early proteins are found to interact with mitochondria (Chandel et al., 2020;Cruz-Gregorio et al., 2020). For the virus to complete its life cycle increased energy demands must be met in HPV infected cells. In more recent studies it was shown that particularly the E6 oncoprotein of high-risk alphaHPV upregulate the mitochondrial metabolism, thus accomplishing increasing amounts of a vast number of mitochondrial complexes (Cruz-Gregorio et al., 2019) which then contribute to ramping up energy production within the mitochondria. In this thesis (Kirschberg et al., 2020) we now identified another direct HPV8-E7 protein-interaction partner, namely the mitochondrial ATP synthase F1 subunit beta (ATP5B), which catalyzes the production of adenosine triphosphate (ATP). We discovered that not only HPV8-E7, but also the E7 proteins of the alphaHPV types HPV11 and HPV

16 interact with ATP5B. Of note, total ATP5B protein levels were not altered *in vitro*. The fact that the invasion deficient mutant HPV8-E7^{L23A} binds weaker to ATP5B allows for the tempting speculation that residues around the mutated L23 might additionally – to some extent – be involved in HPV8-E7 binding to ATP5B. This observation may simultaneously help explaining why HPV8-E7^{L23A} is also inferior in respect to enhancing keratinocyte proliferation and why it is additionally invasion-deficient. Subsequent co-localization experiments confirmed the ability of E7 to co-localize with ATP5B at the mitochondria. This binding may represent a conserved feature among different E7 proteins, even though the mechanism by which mitochondrial entry and interaction with the mitochondrial complexes is unknown. The presence of a TOMM20 recognition motif in HPV8-E7, identified in-silico using the online tool MitoFates (Fukasawa et al., 2015). TOMM20 is part of a mitochondrial pore complex and functions as a “gatekeeper” that facilitates protein entry into the mitochondria (Baker et al., 2007) and may thus explain how at least HPV8-E7 manages to enter the mitochondria. A possible delineation for how the HPV11-E7 and HPV16-E16 proteins enter the mitochondria could be – that they all carry a positively charged amphiphilic region located near their N-terminus that may contribute to facilitating E7 entry into the mitochondria as observed in comparative MitoFates analyses for the E7 proteins of the HPV types included in this publication (Fig. 7).

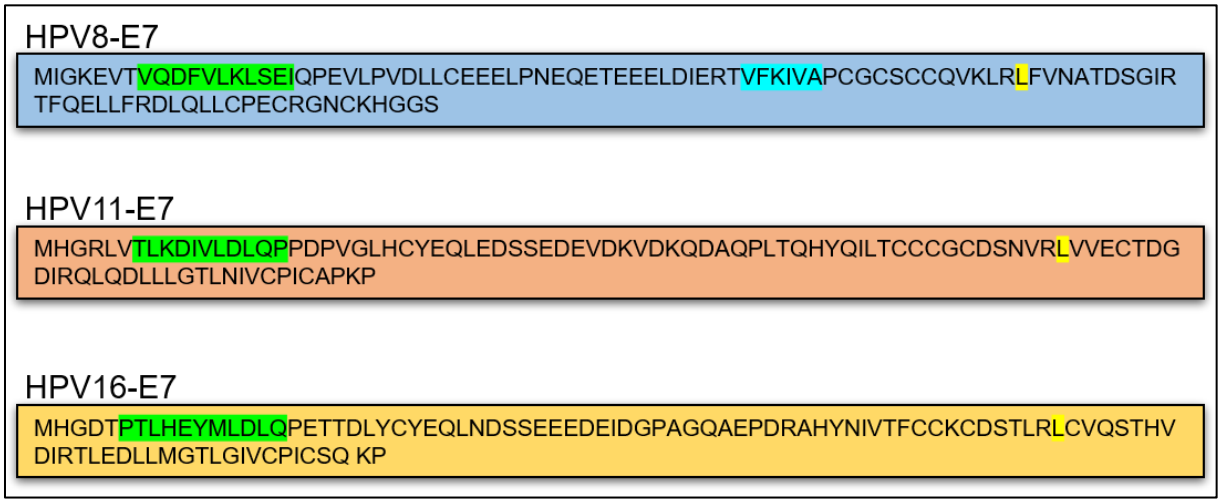

Fig. 7 Schematic representation of the HPV8, HPV11 and HPV16-E7 amino acid sequences adapted from MitoFates analyses probing for mitochondrial recognition sites. HPV8-E7 contains a predicted TOMM20 binding motif (underlaid in turquoise) which both HPV11 and HPV16 lack. TOMM20 fulfills the role of a gate-keeper at mitochondrial pores. Interaction of HPV E7 proteins with mitochondrial pore complexes may thus allow for E7 entry into host mitochondria, in the case of HPV8-E7 by a possible interaction with TOMM20 (underlaid in turquoise). All studied E7 proteins contain an amphicillic region with a length of 11 amino acids (underlaid in green) at their N-terminus, yet with no conserved sequence identity. In addition, they all contain a mitochondrial processing peptidase cleavage site (underlaid in yellow) which may imply that only a truncated E7 protein enters the mitochondria and upregulates mitochondrial activity.

Since the low-risk HPV11-E7 protein also binds to ATB5B this could suggest that E7 binding to ATP5B may be a prerequisite for the creation of a proliferation-promoting environment, which is essential for the completion of viral amplification and could also play a role in driving carcinogenic processes. The observation that the E7 protein interacts with ATP5B also sparked the idea that this may lead to exerting control over the mitochondrial energy production. To see if this may be the case, studies on both glycolytic activity and mitochondrial respiration were performed. As far as glycolysis is concerned, we saw that it was almost completely abolished in HPV8-E7 and HPV16-E7 positive cells compared to control keratinocytes. This was a very intriguing observation in light of the fact that most tumors seemingly re-purpose mitochondria to provide building blocks required for cancer growth and predominantly employ glycolysis to meet their energy demands instead, particularly in the hypoxic areas of solid tumors (Porporato et al., 2018). It was a ground-shattering and highly unexpected result that HPV8-E7 and HPV16-E7 drastically increased the spare respiratory capacity of mitochondria (i.e., the maximum amount of ATP that could be produced was much higher), with HPV16-E7 doing so with much greater efficiency than HPV8-E7. This adds further credence to the theory that E7 proteins achieve control over the mitochondrial energy metabolism.

Our own data regarding the metabolic changes strongly imply that HPV infected cells are able to hijack the host cell energy production to meet their own needs by boosting ATP synthase activity. As the virus life cycle is coupled with the differentiation status and HPV requires proliferating cells to maintain progression of the infection these changes may fuel a) the energy demands to fulfil viral replication and thus achieve completion of the viral life cycle. However, when these changes become de-regulated this may also provide the required fuel for hyperproliferation and subsequent carcinogenesis. In addition to the *in vitro* experiments, we next characterized tissue samples originating from HPV16-positive as well as HPV-negative OPSCC patient samples in order to arrive at an *in vitro-in vivo* correlation and translation to the clinical outcome. The employed immunohistochemical stainings revealed only moderate in total ATP5B levels in patient samples from HPV-negative OPSCC patients. Most strikingly though, we observed a significant rise in ATP5B expression in HPV16-positive OPSCC. Interestingly, changes in ATP5B expression had no effect on overall survival in HPV-negative OPSCC patients, whereas there was a highly statistically significant correlation between high ATP5B expression accompanied by a more

favorable outcome in the HPV16-positive OPSCC patient collective. Having said that it is unknown why the mitochondrial metabolism is so strongly favored in HPV-positive cells. In previous works it had been shown that HPV-negative head and neck squamous cell carcinoma (HNSCC) strongly prefer glycolysis rather than oxidative phosphorylation (OXPHOS). In contrast, HPV-positive HNSCC strongly employ OXPHOS to generate energy (Hübbers and Akgül, 2015;Mims et al., 2015;Jung et al., 2017;Cruz-Gregorio et al., 2019). For instance, Cruz-Gregorio et al. recently showed that HPV16 also increases protein levels of other subunits of the mitochondrial complex (namely complex I-IV) as well as the ATP synthase, which leads to larger mitochondria with increased mass. Also, the E6 oncoprotein increases basal respiration, which is accompanied by more reactive oxygen species leakage, resulting in more oxidative stress and thus DNA damage without an obvious effect on ATP-linked mitochondrial activity (Cruz-Gregorio et al., 2019). From their results the authors concluded that this may partially help to explain why alphaHPV-positive OPSCC are also more susceptible to radio-chemo therapy, as the oxidative stress and the resulting DNA damage may lead to an increased susceptibility to ionizing radiation.

Our data regarding the E7 protein add more weight to this theory as we could show that already the mere presence of the E7 protein was already enough to increase ATP production – at the expense of higher proton leakage – which in turn likely contributes to the previously observed oxidative stress in the aforementioned study. Furthermore, this would also help to explain why HPV-positive OPSCC patients with high ATP5B protein levels have a much better prognosis and seemingly better clinical outcome as mentioned above. Since all HPV early proteins have the potential to the mitochondria future studies should analyze mitochondrial function / dysfunction in the presence of multiple early proteins as these results would be very relevant for the clinical setting for the development of prognostic markers and quite possibly even the development of novel drug targets to treat HPV-mediated cancers.

Publication no. 5: (Akgül et al., 2019)

As already hinted at in above paragraph the research on cell transforming activities of HPV is up-to-date mainly characterized in cells the HPV early proteins individually. In line with this approach previous works from our group had already shown that HPV8-E7-mediated mediates hyperproliferation of keratinocytes and allows these cells to overcome p16 induced cell cycle arrest (Akgül et al., 2005a;Akgül et al., 2007;Westphal

et al., 2009). In this study we performed research on cell cycle regulators and thus identified CHK1 as a novel target of HPV8-E7. However, CHK1 deregulation by E7 occurs only in the presence of E6. Therefore, we conducted further experiments and thus provided additional evidence *in vivo* showing that CHK1 is not only abrogated in skin tumors of K14-HPV8-CER transgenic mice but also in 3D skin cultures of E6E7 co-expressing human keratinocytes. In subsequent experiments we could exclude that transcriptional control and ubiquitin-mediated proteasomal degradation is utilized to degrade CHK1. Most interestingly we identified that autophagy is hijacked by E6E7 and used to degrade CHK1, a mechanism that had thus far been unknown to be targeted by betaHPV. Most intriguingly we identified that CHK1 expression was shadowed by the simultaneous expression of the autophagy marker LC3B (microtubule-associated proteins 1A/1B light chain 3B). Furthermore, when the autophagic flux was suppressed *in vitro* using Bafilomycin A1, CHK1 levels were restored which was paralleled by an accumulation of LC3B. This observation highly implicates that autophagy is targeted by HPV8-E6E7 to reduce CHK1 levels. In the past our group had already shown that, following UV exposure, CHK1 phosphorylation was negatively impacted by the HPV8-E6 oncoprotein while total CHK1 protein levels remained unaffected (Hufbauer et al., 2015). In the field it is widely accepted that a critical step in the pathogenesis of betaHPV induced malignancies is the suppression of host cell death and DNA damage repair pathways following UV-irradiation (Hufbauer et al., 2015;Wendel and Wallace, 2017). In healthy human skin autophagy is a physiological process which plays a critical role in healthy epidermal development and subsequent differentiation of keratinocytes. The human epidermis is a multilayer structure which is regularly replenished by keratinocytes originating from the basal cell layer as they divide and differentiate while migrating upwards to the cornified region of the skin. The proliferating cells of the basal layer are a heterogenous mix comprised of stem cells as well as differentiating cells with a restricted ability for self-renewal (Watt, 1998;Akinduro et al., 2016). Upon reaching the granular layer keratinocytes start losing their organelles and begin expression of structural proteins unique for terminal differentiation, which is accompanied by a flattening of these cells (called corneocytes) and their eventual shedding from the skin. Autophagy is most commonly described as a number of cellular processes which ultimately lead to the lysosomal degradation of cytoplasmic segments (Levine and Klionsky, 2004;Shintani and Klionsky, 2004;Mizushima, 2007;Klionsky et al., 2016). In the context of keratinocytes, the term

autophagy has thus far been portrayed as a mechanism for a cell to undergo senescent cell death (Gosselin et al., 2009;Deruy et al., 2010), However, it is also considered to have a pro-survival function as autophagy helps in the protection from UV-damage (Yang et al., 2012;Qiang et al., 2013;Zhao et al., 2013b;Akinduro et al., 2016). There are two proteins that are of key importance in regulating autophagic processes a) the protein kinase B (PKB, also known as AKT) and b) the mammalian target of rapamycin complex 1 (mTORC1). They are part of the AKT/mTORC1 pathway, which promotes anabolic reactions like protein synthesis and suppresses autophagy if there is a surplus of nutrients (Ravikumar et al., 2004). As such, the autophagosome marker LC3 is predominantly produced in the granular layer of the skin. Inhibition of mTORC1 leads to elevated LC3 levels and a switch from LC3A to LC3B, which is therefore also a feasible autophagy marker. The distinct epidermal autophagy marker expression pattern within the granular layer strongly implies that this process is of pivotal importance for the terminal differentiation of keratinocytes (Akinduro et al., 2016). However, as the HPV replication cycle is also dependent on the differentiation status of the infected epithelium, as mentioned before, we hypothesized that the ability to interfere with autophagic processes may be needed for viral amplification as autophagy is also known to be an infection defense (Puleston and Simon, 2014).

Also, it is interesting to note that autophagy deficiency has a negative impact on UV-induced DNA damage repair processes (Qiang et al., 2013;Yang et al., 2013;Zhao et al., 2013a;Akinduro et al., 2016;Qiang et al., 2016). Inhibition of CHK1 as well as autophagic mechanisms may therefore make cells susceptible for the accumulation of such UV-induced DNA damage. Our study unravels the pivotal importance of autophagy as a regulatory mechanism, which is in turn hijacked by HPV8 to control CHK1 protein levels. This event may very well play a significant role in completion of the viral life cycle, epithelial homeostasis and most importantly, from a clinical standpoint, also carcinogenic processes. Up to now research on autophagy in the context of HPV infection has been studied in more detail for the alphaHPV types. It seems that suppression or activation is dependent on the infection stage. In the initial stage, autophagy is suppressed which is in line with the fact that autophagy is also utilized as a defense mechanism against infection. However, during tumorigenesis autophagy is reactivated to provide the required energy for tumor progression (Lin and Chen, 2018;Aranda-Rivera et al., 2020).

Having said that, to get a more concise understanding regarding the exact mechanism by which HPV infected human skin might suppress CHK1 expression more detailed or focused follow-up studies would be needed, such as proteomic analyses with extracts from E6E7 cells in the presence or absence of autophagy inhibitors.

CONCLUSION AND OUTLOOK:

From the results of this thesis, it is self-evident that the E7 protein of HPV8 is a multi-faceted protein that exerts control over several pathways and influences the host cell on multiple levels. This also includes transcriptome and proteome de-regulation as well as alterations of various major cellular regulators that may very well trigger carcinogenic processes. However, when these oncogenes become aberrantly expressed in immunocompromised individuals this may lead to malignant transformation events. It is logical to presume that all these novel targets identified in my thesis, and previously identified known E7 interaction partners must contribute to the completion of the viral life cycle in some way, which needs to be explored in further studies. Since these oncoproteins play a key role in HPV malignancies, a deeper understanding of how they are expressed (Iftner et al., 1990;Zhao et al., 2019), their functions and structural characterization will likely also contribute to further extend our knowledge on their oncogenic functions.

In current translational research projects in our group, we now aim at understanding the epigenetic changes in betaHPV-positive compared to negative human skin cancers. A very recent study characterized such epigenetic changes in AK and SCC using methylome analyses. Importantly, the researchers could demonstrate that AK methylation patterns already displayed common features of cancer methylomes and were highly similar to SCC profiles. Interestingly, this signature was only observed in half of the samples, while the other half showed methylation patterns more closely related to healthy epidermal tissue. Intriguingly, keratin gene methylation patterns identified two distinct subtypes of AK and SCC, one that originated from undifferentiated epidermal stem cells and the other rising from more differentiated keratinocytes (Rodriguez-Paredes et al., 2018). In cooperation with this research group we tested these two distinct tumor groups for the presence / absence of betaHPV. Interestingly, we so far found a strong correlation between epidermal stem cell like tumors with betaHPV, whereas tumors with a more differentiated keratinocyte

methylation profile rather tend to be betaHPV-negative. Future research aiming at also understanding genetic and epigenetic changes of betaHPV-positive and betaHPV-negative cancers will help – in combination with the results from my thesis – to further pinpoint key genetic and metabolic changes in betaHPV-positive tumors in order to correlate these findings with the expression of either early HPV proteins individually or in combination. Precise oncoprotein targeting approaches may then allow for the formulation of novel specialized therapeutic approaches in the treatment of betaHPV-mediated skin tumors in high-risk patients.

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VI. Appendix

6.1 Abbreviations

alphaHPV	HPV of genus alphapapillomavirus
AK	Actinic keratosis
betaHPV	HPV of genus betapapillomavirus
HPV8-CER	Complete early genome region of HPV8
DDR	DNA damage repair
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EV	Epidermodysplasia verruciformis
FN	Fibronectin
gammaHPV	HPV of genus gammapapillomavirus
HNC	Head and neck cancer
HNSCC	Head-and-neck cancer
HPSG	heparan sulfate
HPV	Human papillomavirus
IARC	International Agency for Research on Cancer
K14	Keratin-14
MMP	matrix metalloprotease
MmuPV1	mus musculus mouse papillomavirus 1
NCR	Non-coding region
NMSC	Non-melanoma skin cancer
OBSL1	Obscurin-like 1
OPSCC	Oropharyngeal squamous cell carcinoma
Ori	origin of replication
OSC	Organotypic skin culture
OTR	Organ transplant recipient
OXPHOS	Oxidative phosphorylation
PV	Papillomavirus
RT-qPCR	Reverse transcription-quantitative PCR
SCC	Squamous cell carcinoma
SFK	Src family cytoplasmic tyrosine kinases

6.2 Figure legends:

6.3 Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Die in Unterpunkt III und im Folgenden aufgeführten Publikationen sind Gegenstand der vorliegenden Dissertation dieser kumulativen Promotion:

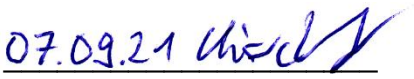
Novel insights into cellular changes in HPV8-E7 positive keratinocytes: a transcriptomic and proteomic analysis. **Kirschberg M**, Syed AS, Dönmez HG, Heuser S, Wilbrand-Hennes A, Hufbauer M, Akgül B. 2021, Front. Microbiol. 12:672201.

ATP synthase modulation leads to an increase of spare respiratory capacity in HPV-associated cancers. **Kirschberg M**, Heuser S, Marcuzzi GP, Hufbauer M, Seeger JM, Đukić A, Tomaić V, Majewski S, Wagner S, Wittekindt C, Würdemann N, Klussmann JP, Quaas A, Kashkar H, Akgül A. Scientific Rep. 2020; 10:17339

HPV8 activates cellular gene expression mainly through Sp1/3 binding sites. **Kirschberg M***, Heuser S*, Syed AS, Steger G, Majewski S, Hufbauer M, Akgül B. Virology. 2019; 535:136-143. *Equal contribution

BetaHPV E6 and E7 colocalize with NuMa in dividing keratinocytes. Oswald E*, **Kirschberg M***, Aubin F, Alonso A, Hufbauer M, Akgül B, Auvinen E. Virus Genes. 2019; 55:600–609. *Equal contribution

Human papillomavirus type 8 oncoproteins E6 and E7 cooperation downregulation of the cellular checkpoint kinase-1. Akgül B, **Kirschberg M**, Storey A, Hufbauer M. Int. J. Cancer. 2019; 145:797-806



(Matthias Kirschberg)

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