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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 beta-papillomavirus (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),

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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

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 deepidermalized 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
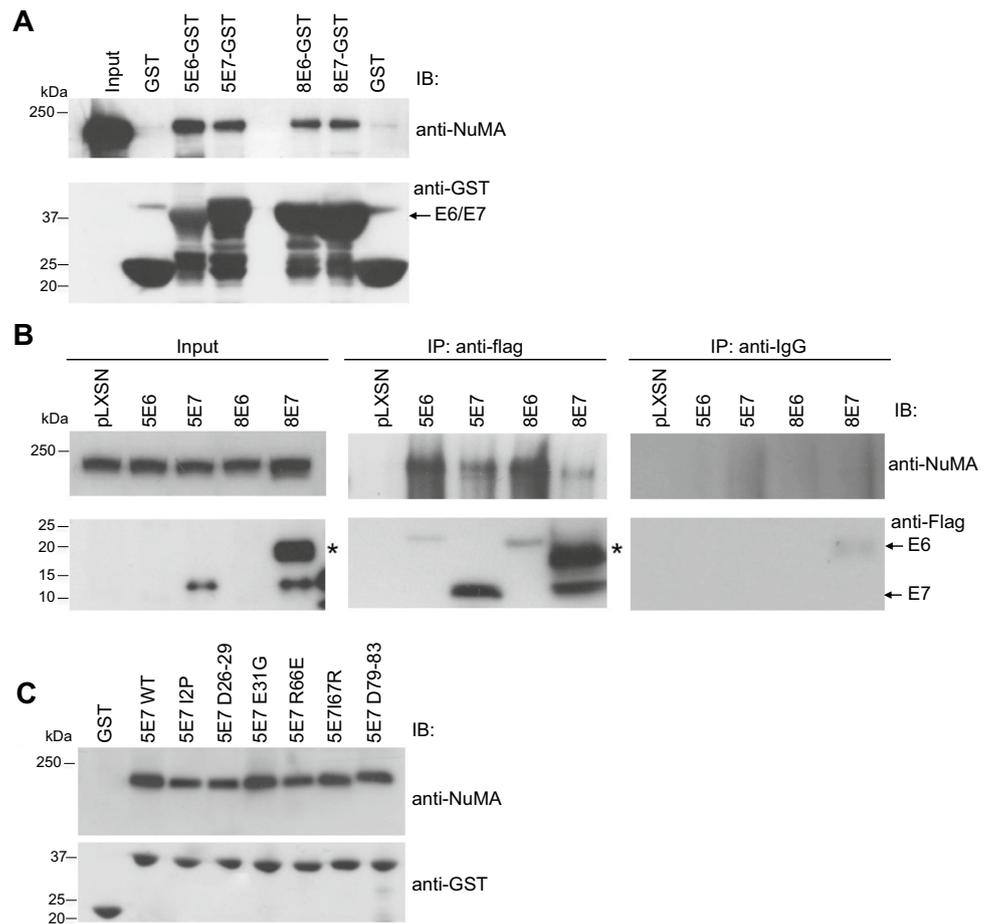
basal cell layer with decreasing staining intensity toward the skin surface. In skin SCC sections from EV patients we detected different staining patterns. For instance, in section EV-a, NuMA is mainly nuclear but also cytoplasmic and restricted to the suprabasal cell layers. Unexpectedly, in the skin 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).

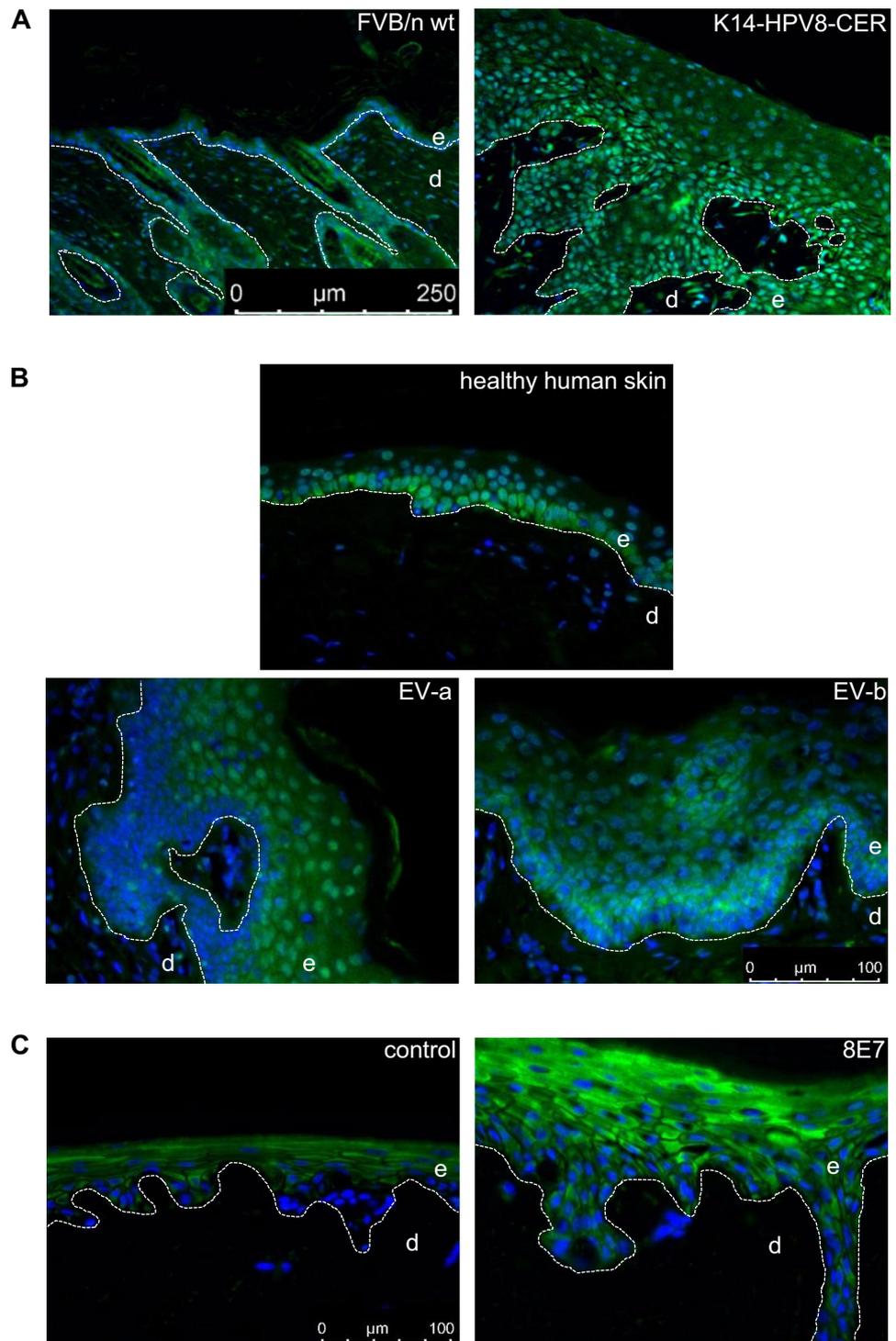## E6 and E7 and NuMA colocalize in interphase

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).
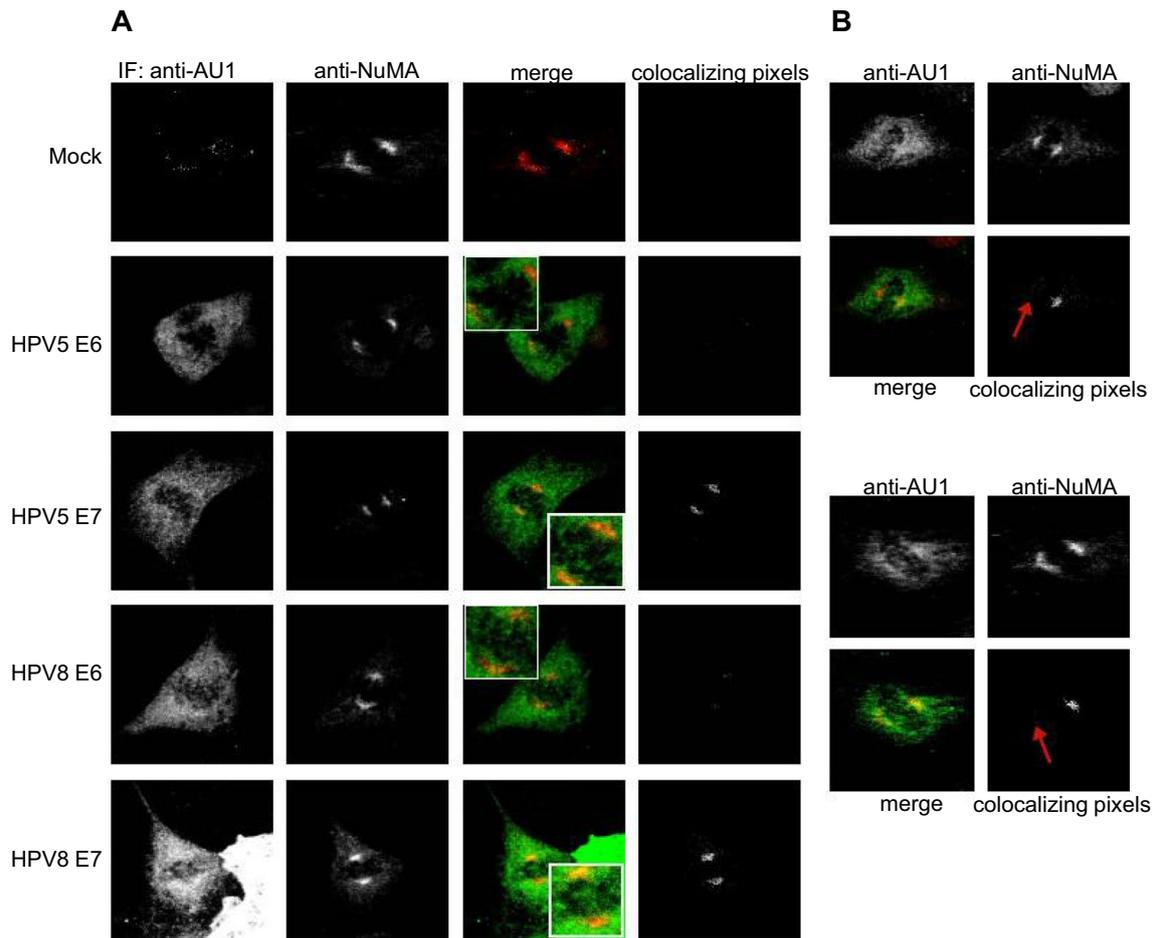
Fig. 3 E6 and E7 and NuMA colocalize in interphase. **a** COS-7 cells were transfected with expression plasmids for HPV5 or HPV8 E6 or E7 provided with AU1 epitope. Fixed cells were stained with antibodies to AU1 (green) or NuMA (red). The images show *z*-axis projections of 15–18 layers. Colocalized pixels were calculated using the Colocalization Highlighter Plugins of ImageJ. Only those pixels which exceeded the cutoff intensity of 100 (8 bit) and whose intensi-ties on both channels showed at least 95% correlation were taken into account. **b** E7 and NuMA colocalize at mitotic spindle poles. Immu-nofluorescence 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: indi-cates 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 inter-ested 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 trans-duced 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 (Sup-plementary Fig. 2a, c). We next treated the transduced PHK cells with nocodazole, a reagent inhibiting polymerization of microtubuli and causing cell cycle arrest through activa-tion 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 popula-tion 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 destabiliza-tion of the mitotic complex of NuMA with dynein and con-sequently 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 onco-genes 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 consider-able. 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 estab-lished [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 pro-teins are also capable of binding NuMA. However, the inter-acting 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 orches-trates correct organization of microtubules at centrosomes and proper chromosome alignment in preparation of cell division. Contrary to HPV16 E7 [39, 40], only a small pro-portion of betaHPV E6E7 expressing cells display extra cen-trosomes, suggesting that there is only a minor interference with centrosome separation upon betaHPV E6E7 binding to
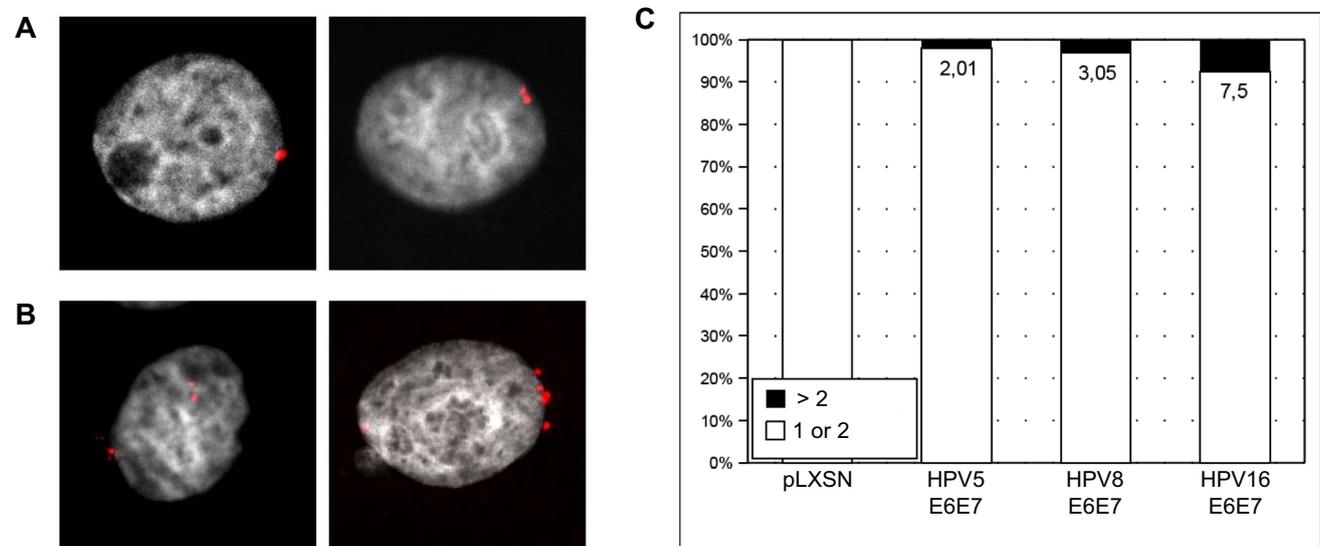

Fig. 4 PHK were transduced with pLXSN vector, or constructs expressing E6 and E7 of either HPV5, HPV8 or HPV16, and the cells were stained for pericentrin (red). a Left image: control; right image: HPV5-E6E7. b Left image: HPV8-E6E7; right image: HPV16-E6E7. c Proportions in percentages of HPV5, HPV8 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.

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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