Virus-host protein-protein interactions between human papillomavirus 16 E6 A1 and D2/D3 sub-lineages: variances and similarities

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Abstract

High-risk strains of human papillomavirus are causative agents for cervical and other mucosal cancers with type 16 being the most frequent. Compared to the European Prototype (A1 denoted "EP"), the Asian-American (D2/D3 denoted "AA") sub-lineage or "variant" was reported to have increased abilities to promote carcinogenesis. Few, if any global interactome studies, have looked at protein-protein interactions (PPIs) between host proteins and variants of the key transforming E6 protein. We applied a primary human foreskin keratinocyte model transduced with EP and AA variant E6 genes and co-immunoprecipitated expressed E6 proteins along with interacting cellular proteins to potentially detect diversity in virus-host relationships. We reasoned that due to single nucleotide polymorphisms, AAE6 and EPE6 may have unique PPIs with host cellular proteins—conferring gain or loss of function—resulting in varied abilities to promote carcinogenesis. Using liquid chromatography-mass spectrometry and stringent interactor selection based on the number of peptides, we identified 25 candidates: 6 unique to AAE6 and EPE6, respectively along with 13 common E6 targets between AAE6 and EPE6. We also applied a more inclusive process based on pathway selection and discovered 171 target proteins: 90 unique AAE6 and 61 unique EPE6 along with 20 common E6 targets between the two sub-lineages. Interpretations for both approaches were made drawing from different databases such as UniProt, BioGRID and Reactome. The detected E6 targets were found to be implicated in deregulating a variety of cellular functions such as DNA replication and repair, energetics and hypoxia, mitogen-activated protein kinases/extracellular signal-regulated kinases signaling, tumour suppression and immune response, as well as pathways hijacked from embryogenesis and wound healing to drive cancer development. Validation experiments such as reverse co-

immunoprecipitation and RNA interference are required to substantiate these findings. Here, we provide an unprecedented resource for new research questions and hypotheses testing in HR HPV biology. If variant-specific interactions are verified, their targeted disruption may potentially yield new options for personalized cancer treatment strategies.

1. Introduction

HPV16 sub-lineages

Human papillomaviruses (HPVs) are double-stranded DNA viruses which infect the keratinocytes of skin and mucosal tissues (zur Hausen 2002). Persistent infection with any of the high-risk (HR) types is now implicated in causing almost every case of cervical cancer (Wallboomers et al. 1999, Crow 2012). HR HPVs also have an aetiological association with cancers of other anogenital regions as well as a growing number of head and neck cancers (Garbuglia 2014). HPV16 (a member of *Alphapapillomavirus-9* species) is one of the most common HR types (Crow 2012) and comprises four sub-lineages or "variants": A (European prototype sub-lineages A1/A3/A3 and the Asian sub-lineage A4), B (African-1 sub-lineages B1/B2), C (African-2) and D (North American sub-lineage D1 and Asian-American sub-lineages D2/D3) (Chen et al. 2005, Burk et al. 2013).

We and other groups have been studying three HPV16 E6 protein variants (Figure 1): European prototype (EP), European-T350G (E-T350G: has an L83V single nucleotide polymorphism (SNP) in the E6 protein) and Asian-American (AA: has Q14H/H78Y/L83V SNPs in the E6 protein) (Zehbe et al. 1998, 2009, Zacapala-Gómez et al. 2016, Hochmann et al. 2019). Epidemiological evidence has demonstrated that the AA variant is associated with a higher risk

for developing high grade cervical lesions and cervical cancer, as well as with an earlier cancer onset (Xi et al. 1997, Villa et al. 2000, Berumen et al. 2001, Xi et al. 2007). The epidemiological evidence surrounding the E-T350G variant is more complex, showing population-specific risk (Togtema et al. 2015 and refs therein, Zhang et al. 2016). Variant functional investigations recently completed by us have demonstrated that EP, E-T350G, or AAE6 retrovirally transduced into primary human foreskin keratinocytes (PHFKS) is able to immortalize these cells even in the absence of E7 (Niccoli et al. 2012, Togtema et al. 2015). While E-T350G and AAE6 promoted quicker cell growth and an earlier escape from growth crises than EPE6, only AAE6-trandsuced cells were able to form viable colonies in soft agar and demonstrate the ability to migrate and become invasive. Similar results were seen when E7 was also present (Richard et al. 2010), except that E-T350G was also then able to form viable colonies in the soft agar transformation assay (Sichero et al. 2012). When keratinocytes were grown in 3D raft cultures, E-T350G and AAE6 caused higher grade dysplasia with more severe abrogation of differentiation patterns than EPE6 (Zehbe et al. 2009, Jackson et al. 2014). We found that AAE6 elevated the levels of glycolytic enzymes related to the Warburg effect (Richard et al. 2010) and that of hypoxia inducible factor 1 alpha (HIF-1α) (Cuninghame et al. 2017), possibly contributing to AAE6's enhanced proliferative abilities. The deregulation of HIF-1α was caused by AAE6's ability to augment mitogen-activated protein kinase/extracellular related kinase (MAPK/ERK) signaling. It has also been shown that E-T350G differentially affects the amount of MAPK and phosphoinositide 3-kinases (PI3K)/activated kinase tyrosine (AKT) signaling (Chakrabarti et al. 2004, Sichero et al. 2012, Hochmann et al. 2016), binding to calcium-induced binding protein and disks large homologue (hDlg) (Lichtig et al. 2006) as well as E-cadherin protein levels (Togtema et al. 2015).

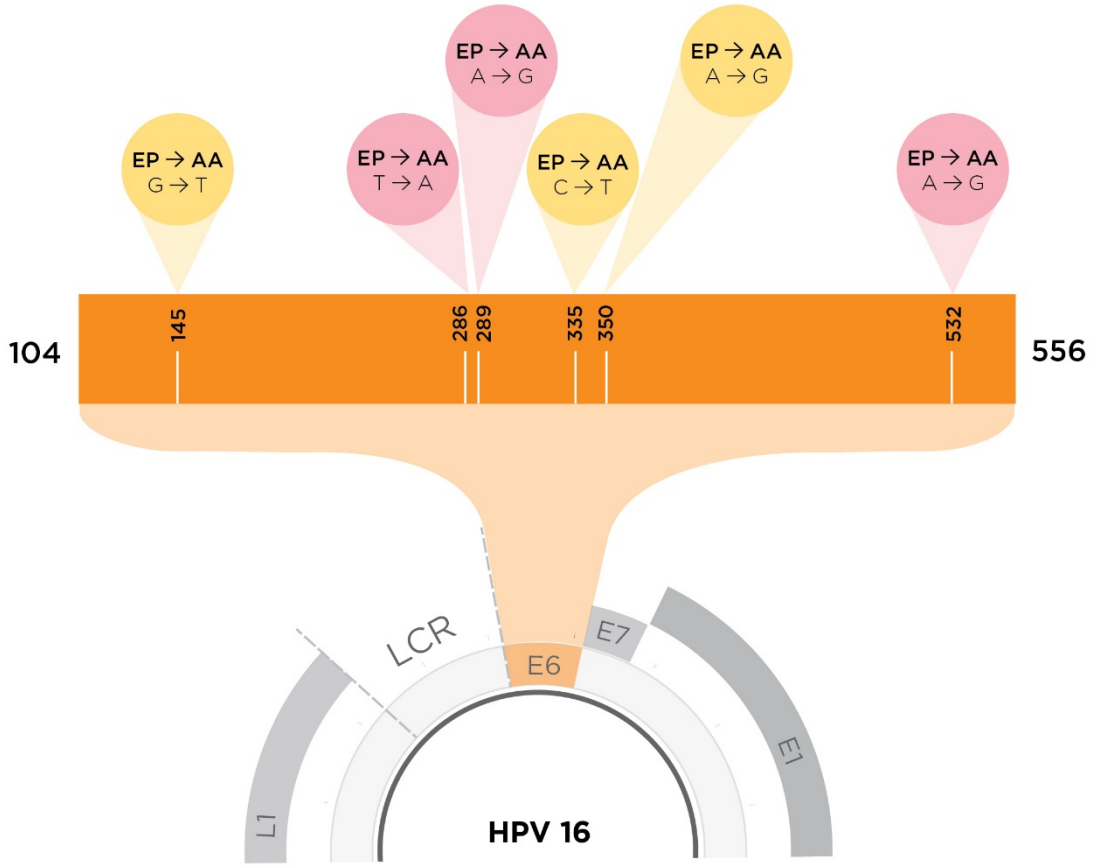


Figure 1 — To scale depiction of HPV16 E6 SNPs between AAE6 and EPE6. The yellow cones indicate a SNP that results in an amino acid change (missense mutation), whereas the pink cones indicate no amino acid changes at that particular site (nonsense mutation). SNPs resulting in amino acid changes from EPE6 to AAE6 (Q14H, H78Y, and L83V) are found at nucleotide (NT) positions G145T, C335T, and T350G. SNPs that do not result in any change in amino acids are found at NT positions: T286A, A289G, and G532A (Zehbe et al. 1998).

To elucidate the mechanisms behind the above functional observations, Zacapala-Gómez et al. (2016) completed a global transcriptome study of E6 variants expressed in C33A cells and found 387 differently expressed genes compared to cells transfected with EPE6. The genes involved were related to cellular processes such as adhesion, angiogenesis, apoptosis, differentiation, cell cycle, proliferation, transcription and protein translation. Enrichment analysis of the Affymetrix data showed an over-representation of more than 1.5-fold for immunological

processes for AAE6 compared to EPE6. The use of carcinogenic C33A cells may already have had underlying alterations in the expression of these pathways prior to the introduction of the E6 protein which could impact these findings. A whole HPV16 genome sequencing study using 7116 pooled patient samples reported that the D2/3 (AA) as well as A3/4 sub-lineages show increased cervical cancer risk compared to A1 [EP of the current study] (Clifford et al. 2019). When taken together, these results led to the hypothesis that amino acid changes of the variant E6 proteins may allow them to bind a unique set of host proteins compared to EPE6, rather than the variant proteins merely interacting in different amounts with the same set of host proteins.

HPV16 E6 functions and host cell targets

For the past 30 years, researchers have extensively studied how the E6 protein deregulates and transforms host cells (reviewed in Klingelhutz and Roman 2012, Vande Pol and Klingelhutz 2013 and references therein). HPV16 E6 confers viral persistence by down-regulating type I interferons (IFNs) alpha, beta (Ronco et al. 1998, Nees et al. 2001), kappa (Rincon-Orozco et al. 2009, DeCarlo et al. 2010, Reiser et al. 2011) as well as the type II IFN gamma (de Gruijl et al. 1999). Studies have shown that E6 prevents retention of Langerhans cells in infected epithelium through the down-regulation of E(pithelial)-cadherin (Matthews et al. 2003). E6 induces cellular immortalization through upregulation of hTERT, the catalytic subunit of the enzyme telomerase (Klingelhutz et al. 1996, Veldman et al. 2003, Gewin et al. 2004, Katzenellenbogen et al. 2007), allowing cells to divide for an indefinite number of passages. Concurrently, E6 degrades the tumour suppressor protein P53 by forming a complex with it and the cellular ubiquitin ligase E6AP (Scheffner et al. 1993, Zanier et al. 2012), thus preventing cells from entering apoptosis upon the subsequent accumulation of genetic changes. A transformed

cellular phenotype is promoted through E6's ability to bind PDZ (first letters of the first three proteins sharing the domain: post synaptic density protein, Drosophila disc large tumour suppressor and zonula occludens-1 protein) domain-containing proteins through its C-terminus such as membrane-associated guanylate kinase (MAGI)-1 (Kranjec and Banks 2011), MAGI-2, MAGI-3 (Thomas et al. 2002), h(uman) Scribble (Nakagawa and Huibregtse 2000) and hDlg (Kiyono et al. 1997, Gardiol et al. 1999), disrupting cell-cell signalling, cellular adhesion and cell polarity.

The most likely underlying mechanism for the described E6 functions is protein-protein interaction (PPI) between E6 and host cellular targets. We have summarized the findings of previously reported HPV16 E6 interactors (n=50 to date) in Supplemental Table 1 with hyperlinked references therein. The first study encountered in our survey was that by Werness et al. (1990) and the last one that by Oliveira et al. (2018). Most commonly used methods for PPIs were the yeast two hybrid (Y2H) screen, glutathione S-transferase (GST) pulldown, immunoprecipitation (IP) and co-IP. Six papers also reported some form of mass spectrometry (MS) (Nakagawa and Huibregtse 2000, Jeong et al. 2007, Jing et al. 2007, Katzenellenbogen et al. 2007, Vos et al. 2009, White et al. 2012). There is some confusion about protein nomenclature: in the first column (left), we refer to an *alias* if the Uniprot protein nomenclature (The UniProt Consortium 2019) differs from that used in the publication, *e.g.* in the title and/or abstract; in the other columns, we use the nomenclature adopted by the respective publication. When looking at the binding mechanism, we considered the domain of the targeted protein regarding its amino acid sequence, region (N- or C-terminus) where this information was available. Using the UniProt database (The UniProt Consortium 2019), we have collectively interpreted the data of all 50

binders together in the light of biological functions. Viral processes and immune response, mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) cascade as well as Wnt (portmanteau of Wingless and int-1, Nusse et al. 1991) and Notch ("notches at the Drosophila wing margin", Artavanis-Tsakonas et al. 1999) signaling pathways—both highly conserved in evolution and embryogenesis-related, were mostly targeted followed by tumour suppressor proteins, DNA damage and repair, apoptosis, adhesion, immortalization and transformation, cell cycle and proliferation, and transcription. In line with these observations, a recent publication dissecting viral associations in patient specimen of the Pan-Cancer Analysis of Whole Genomes Consortium, impaired antiviral defence mechanisms were found to be the driving force for HPV16-related malignancies including cervical, bladder and head and neck cancers (Zapatka et al. 2020).

Notably, most if not all interaction experiments of the 50 known binders to date were conducted not specifying which HPV16 E6 sub-lineage was used other than "wildtype" or "prototype" with or without artificial mutants. Here we started filling this gap by investigating two common, naturally occurring E6 variants of the A1 (EPE6) and D2/D3 (AAE6) sub-lineages because we hypothesized that differential PPIs between E6 and host cell proteins matter. Our methodological approach included primary human foreskin keratinocytes—naturally targeted by HPV, as well as state-of-the-art liquid chromatography-MS for virus-host PPI analysis and regularly-curated, freely-accessible databases. The obtained results will facilitate many future studies in HR HPV biology and may prove useful for anti-cancer treatment strategies.

2. Methodological Approach

Wet lab methods

Wet lab methods such as cell culture, cell lysis, Western blot and co-immunoprecipitation (co-IP) used in this investigation are described in detail in Supplemental Information and Supplemental Figures 1 and 2. The most challenging step in optimizing co-IP was protein elution, which was limited by several factors: compatibility in downstream liquid chromatography tandem mass spectrometry (LC-MS/MS) applications, the effectiveness of removing target proteins and minimizing antibody leeching. Elution methods described by other groups (White et al. 2012, Grace and Munger 2017) failed to elute any detectable level of E6. The use of acidic elution used in this paper proved not only to be most effective at elution but also was compatible for LC-MS/MS. Although E6 is a particularly difficult protein to detect in studies involving LC-MS/MS studies (White et al. 2012) we were successful in detecting AAE6 in one of the two trials.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Two independent trials were performed with AAE6-HA, EPE6-HA and HA vehicle-transduced primary human foreskin keratinocytes (PHFK-HA) treated with the proteasome inhibitor MG132-DMSO or DMSO only, to yield the highest possible number of E6-binding partners (White et al. 2012). Samples were shipped to the Harvard Center for Mass Spectrometry (HCMS), Cambridge, MA, USA and processed as a paid service on demand. To reduce samples, 2.0 µL of 20 mM Tris(2-carboxyethyl) phosphine hydrochloride solution [pH 1.2-1.5] (Sigma, Cat# 75259-1G) in 50 mM triethylammonium bicarbonate (TEAB) [pH 8.0] (TEAB, Sigma, Cat# T7408) buffer were mixed to 20 µL of sample. Samples were incubated at 37°C for 1 hour and subsequently warmed to room temperature, vortexed and pulse-centrifuged to ensure samples

remained at the bottom of each tube. Next, 2.2 µL of freshly prepared 40 mM iodoacetamide (Sigma, Cat# I1149-5G) in 50 mM TEAB was added to each sample for alkylation. Samples with added iodoacetamide were covered with tin foil and incubated at room temperature for 1 hour. After successful reduction and alkylation of samples, trypsin (1:50-1:100 [w/w], Fisher Sci., Cat# SH3023602) was added and incubated at 37°C for 16 hours overnight. After incubation, samples had 1 µL of formic acid (Sigma, Cat# 56302-50ML) thoroughly vortexed and placed into a nanoACQUITY ultra performance liquid chromatography (UPLC) system (Waters, Cat# WTF-ARC-2998) to begin LC-MS/MS protein identification. The mass spectrometer used for all LC-MS/MS tests was an Orbitrap Elite™ Hybrid Ion Trap Orbitrap Mass Spectrometer (ThermoFisher Sci., Cat# IQLAAEGAAPFADBMAZQ). After each sample was placed into the mass spectrometer the spectra obtained were input into Proteome Discoverer version: 2.4.0.292. Three databases were used for identification of proteins: Uniprot Human Proteome Database; a custom-curated common contaminant database and a user-defined database containing the amino acid sequences for HPV16E6 (AAE6 and EPE6). Identified proteins were emailed as an original "raw" heatmap using Microsoft Excel.

Databases

To analyze relationships between proteins identified by LC-MS/MS, we used three regularly-curated and freely-accessible databases: The Universal Protein Resource (UniProt) (The UniProt Consortium 2019) for molecular functions and biological processes, the Biological General Repository for Interaction Datasets (BioGRID) (Oughtred et al. 2019) for PPI analysis and the Reactome Pathway Database (Reactome) (Jassal et al. 2020) to map E6 protein binders to the

biological pathway in which they are involved. All three databases are continuously updated and monitored to provide users with the latest, available information including relevant references.

UniProt provides an accession number for each protein, which can be inserted into Reactome or BioGRID. Reactome is hierarchically organized into main pathways which are dissected by multiple-degree sub-pathways. When retrieving the pathways targeted by a protein of interest, the entire pathway hierarchy is simultaneously "queried". Obtained data need to be analysed cautiously since several in-put proteins could be listed in the same main pathway yet be involved in sub-pathways unrelated to each other further down the hierarchy. As well, some of the identified proteins may not match with any pathways existing in the Reactome database. Reactome generates a ranking of the 25 most significant pathways meaning that these pathways are over-represented compared to others based on their probability value (P-value) and false discovery rate (FDR) (Fabregat et al. 2017). In our context, Reactome was used to discriminate non-specific binders and to determine how cellular pathways are [differentially] targeted by AAE6 vs. EPE6 and BioGRID if proteins of interest were interacting with each other.

Protein filtering post LC-MS/MS

MS contaminants removal

We merged the original HCMS excel files which listed each trial (T1 and T2), condition (DMSO and MG132), E6 variant (AAE6 or EPE6) and PHFK-HA. After removal of contaminants which were due to processing of the samples for MS and labelled *CON* (n=50), we obtained a raw heatmap with a total of 1,584 proteins listed in alphabetical order (Supplemental Table 2). We then cleaned the data from ribosomal proteins (n=95). When performing a Co-IP, proteins binding to the support specifically or non-specifically also bind to their respective partners. Since

ribosomal proteins are non-specific binders, several of their partners may also be present in the samples. We therefore ran them through a Reactome (Fabregat et al. 2017) analysis using all the remaining proteins and further removed proteins involved in pathways of RNA metabolism (n=89) yielding 1,464 remaining proteins (Supplemental Table 3). Throughout, we have adopted the protein abbreviations as defined by UniProt, e.g. P53 for the tumour-suppressor protein 53.

E6 interactors selection

Peptide method

Proteins present in any of the PHFK-HA samples are likely to be contaminants and were therefore removed (n=586; Supplemental Table 3). Two stringent rules denoted the "*Peptide method*" were then used to select potential candidates among the remaining proteins: any EPE6- or AAE6-targeted protein must have a sum of at least 2 peptides in either trial independent of treatment, OR any EPE6- or AAE6-targeted protein must have at least 1 peptide in both trials independent of treatment. This approach left us with 25 cellular protein targets: 13 targeted by both variants and 6 unique to each AAE6 and EPE6 (Supplemental Table 4).

Protein-pathway method

Of the 50 E6 interactors that have been identified by other groups (Supplemental Table 1), we found 7 (P53, UBE3A, MAGI2, MAGI3, BAK CYLD and SIPA1L1) in our raw heatmap with SIPA1L1 only in PHFK-HA (Supplemental Table 1). After the peptide method selection (Supplemental Table 4), only P53 and UBE3A were present due to the fact that most proteins were found with a limited number of peptides or only in one experiment. This is interesting as the 2-peptide selection strategy or selection of proteins identified in more than one trial are commonly accepted procedures in publications (Katzenellenbogen et al. 2007, White et al. 2012).

Based on our current results, this led to several established interactors indeed being missed with these selection strategies. This and the fact that some known E6 binders such as MAGI2 were present with only 1 peptide, in 1 trial and in 1 treatment (Supplemental Table 2), we also designed a more inclusive approach to select additional potential E6 targets. We reasoned that, although it is possible that LC-MS/MS will miss several proteins in a given sample it is far less likely that a whole pathway will be missed. Emphasis was on differences in proteins between 2 groups rather than on peptide abundance alone, i.e. AAE6 versus PHFK-HA, EPE6 versus PHFK-HA and AAE6 versus EPE6. We detected 498 proteins in AAE6, 380 in EPE6 (n=108 overlap) and 586 proteins in PHFK-HA samples (Supplemental Table 3). Each group of proteins was then analysed using Reactome to identify the pathway involving these proteins: 251/498 AAE6-targeted proteins were matched to 861 pathways, 199/380 EPE6-targeted proteins to 794 pathways and 323/586 "unspecific" protein binders in PHFK-HA were matched to 954 pathways. We then compared the pathways obtained for each group and selected those that aligned with AAE6 and/or EPE6 interactors but not with PHFK-HA interactors. We reasoned that pathways only targeted by AAE6 or EPE6 would be most specific leaving us with 90 unique proteins for AAE6, 61 unique proteins for EPE6 and 20 additional proteins common to both, i.e. altogether 171 potential E6 binders (Supplemental Table 5). These proteins were then run independently (all AAE6 interactors and all EPE6 interactors separately) on Reactome to assess which pathways were significantly over-represented for each E6 sub-lineage.

3. Results and Discussion

Peptide method

Data was gathered independently of treatment as announced above (Methodological Approach). The 25 candidates (13 common E6-interacting proteins between AAE6 and EPE6, along with 6 unique AAE6 and 6 unique EPE6 interacting proteins; Figure 2A; Supplemental Table 4) obtained by the Peptide method were screened for functions related to HPV16-related tumourigenesis and immune suppression obtaining a short-list of 7 proteins: 3 were found in both AAE6 and EPE6: E3 ubiquitin-protein ligase trip12 (TRIPC, Q14669), nucleolar GTP-binding protein 2 (NOG2, Q13823), charged multi-vesicular body protein 4b (CHM4B, Q9H444); 3 were only found in AAE6: inositol (INO)80 complex subunit B (IN80B, Q9C086), ribosomal protein S6 kinase alpha-4 (KS6A4, O75676), prokineticin-2 (PROK2, Q9HC23); and interferon-induced GTP-binding protein (MX2, P20592) was only found in EPE6 samples.

AAE6 interactors

IN80B, an ATPase, is part of the ATP-dependent INO80 remodeling complex containing at least 12 other proteins. The INO80 complex has key functions in transcriptional regulation, DNA replication and repair, telomere maintenance and chromosome segregation (Min et al. 2013). In a mouse model of cervical cancer, Hu et al. (2016) showed INO80 to be overexpressed and that, when bound to the Nanog (TÍr na nÓg, the mythical Celtic “Land of the Young”) transcription start site, this transcription factor’s expression was enhanced. Blocking this interaction led to a decrease in proliferation. In addition, Y2H indicated that IN80B interacts with HPV16 E7 and HPV5 E7 (Rozenblatt-Rosen et al. 2012) suggesting that both E6 and E7 could alter the function of IN80B. While IN80B was found to interact with other than the chosen 7 candidates of the peptide

method namely nucleolar complex protein 3 homologue (NOC3L, Q8WTT2), ribosomal RNA processing 12 homologue (RRP12, Q5JTH9) and retinitis pigmentosa 9 protein (RP9, Q8TA86) using Co-IP (Kuroda et al. 2004, Cloutier et al. 2017), no common pathways between them were found with Reactome analysis nor were they listed as constituents of the INO80 complex (Chen et al. 2011). Proximity label MS showed that the Myc (myelocytomatosis) proto-oncogene protein (MYC), a known target of E6 (Mesilaty et al. 1998, Table 1), also interacts with NOC3L and RRP12 (Kalkat et al. 2018). Notably, IN80B and RRP12 were present only in the AAE6 sample, NOC3L was targeted by both E6 variant proteins, whereas RP9 was unique to EPE6. Hence, even if EPE6 does not immunoprecipitate IN80B, EPE6 may alter its functions indirectly. Reactome analysis showed IN80B to be associated mainly with DNA damage and repair pathways and metabolism of proteins (Supplemental Table 6).

KS6A4 (also called mitogen and stress activated protein kinase 2, MSK2) belongs to the 90 kDa ribosomal S6 kinase (RSK) family of serine/threonine-protein kinases consisting of 6 human isoforms RSK1-4 and MSK1-2 which are involved in the regulation of cell proliferation, survival, growth and differentiation (Arthur 2008, Llanos et al. 2013). MSKs act downstream of the rat-sarcoma (Ras)/MAPK/ERK or MAPK kinase/p38 phosphorylation cascade (Hauge and Frödin 2006). They regulate several transcription factors by phosphorylation such as cAMP response element-binding protein (CREB), nuclear factor-κB (NF-κB), activating transcription factor 1 (ATF1) (Llanos et al. 2013). MSKs were suggested to be negative regulators of the innate immune system (Ananieva et al. 2008) as their regulation of CREB and ATF1 control the expression of interleukin-10 and dual specificity protein phosphatase 1. MSK2 is also involved in chromatin remodeling by phosphorylating histone H3 and the high mobility group chromosomal

protein 14 (Llanos et al. 2013). Despite being a stress-response activated kinase, MSK2 has functions under normal conditions that do not involve kinase activity (Llanos et al. 2013). Indeed, MSK2 acts as an inhibitor of constitutively expressed P53 and interacts with P300 thereby blocking P300 activation of P53. MSK2 also binds to the NOXA (damage) promoter inhibiting NOXA transcription by P53. Since MSK2 does not bind P53 or interferes with P53's association with the NOXA promoter, it has been suggested that MSK2 represses NOXA expression by blocking other regulatory elements required for its expression (Llanos et al. 2013). Consistent with its P53 inhibitory functions, MSK2 is degraded during apoptosis (Llanos et al. 2013). In squamous cervical carcinomas, MSK2 is overexpressed and activates paired-box gene 8 (PAX8). How PAX8 is involved in cancer development is not fully characterized, but it is known that PAX8 increases E2F1 expression leading to an increase in tumour formation (Wu et al. 2019). Furthermore, the silencing of MSK2 leads to a decrease in SiHa and HeLa cell proliferation (Wu et al. 2019) and as a control mechanism, pRB interacts with PAX8 limiting E2F1 activation (Li et al. 2011). Interestingly, in a study by the Villa group, it was observed that ERK1/2 phosphorylation of MSK2 was higher in AAE6 compared to EPE6 and E-T350G also called L83V (Hochmann et al. 2016), which resonates well with our current finding that MSK2 was targeted by AAE6 but not EPE6.

We have previously identified increased signaling in the ERK1/2 pathway for AAE6 compared to EPE6 suggesting that this kinase is the only contributing factor to increased hypoxia-inducible factor (HIF)-1α in AAE6 over EPE6 cells in the hypoxic tumour environment (Cuninghame et al. 2017). Interestingly, under hypoxic conditions, NF-κB/p65 is a regulator of HIF-1α gene expression (Yoshida et al. 2013) while the activated MSK2 has been shown to

activate NF-κB/p65 under different stimuli (Vermeulen et al. 2009, Jacks and Koch et al. 2010). Therefore, one could speculate that ERK1/2 activation of MSK2 promotes NF-κB/p65 positive regulation of HIF-1α. Consistent with this notion, KS6A4 was only targeted by AAE6. We conclude that the AAE6 PPI with the KS6A4 kinase may be one of the underlying factors for the differential finding in sugar metabolism between AAE6- and EPE6-transduced PHFKs previously reported by us (Richard et al. 2010; Cuninghame et al. 2017). Notably, Reactome did not yield pathways related to hypoxia with KS6A4 (Supplemental Table 6).

PROK2 is a chemokine-like protein usually expressed by components of the innate immune system such as macrophages with specific roles in host defence and angiogenesis during virus-related cancers (Lauttia et al. 2014, Kurebayashi et al. 2015). Lauttia et al. (2014) observed an increase in PROK2 expression in the epithelium of human Merkel cell carcinoma (MCC)—a rare skin cancer caused by infection with MC polyomavirus (MCpyV) reminiscent of HPV-related cancer biology via the large T antigen and E6, respectively targeting P53. The onset of MCC correlated with an augmentation of tumour-infiltrating macrophages. Using a colorectal cancer cell and mouse model, PROK2 promoted angiogenesis leading to an increase in colon tumour mass (Kurebayashi et al. 2015). Interestingly, PROK2 is known to sequester the promoter of HIF-1 (resonating with our earlier comments above) and to alter extracellular matrix potentially controlling angiogenesis (LeCouter et al. 2001, 2003). PROK2 was found in 7 Reactome pathways mostly related to receptor-mediated signal transduction but not to hypoxia—each associated with AAE6 and EPE6 albeit with different entities (Supplemental Table 6). Taken together, PROK2 may be another interesting AAE6-target as its dual role in host HIF-1 and immune biology may contribute to the “success” of this sub-lineage in tumour development.

EPE6 interactors

Proinflammatory signals is one of the hallmarks of cancer as cells often undergo necrosis recruiting inflammatory cells that in cancers can promote angiogenesis, proliferation and invasiveness (Hanahan & Weinberg 2011). MX2 is a protein involved in innate immune response due to viral infections like human immunodeficiency virus (HIV). One of the main responsibilities of MX2 in HeLa cells is to permit G_1/S cell cycle progression (King et al. 2004). In HPV16-positive cells (W12 cell line), treatment with type I IFN (IFNα/β)-inducible genes including MX2 resulted in the loss of viral episomes (Pett et al. 2006). Based on Y2H experiments, MX2 interacts with the histone-lysine N-methyltransferase EHMT2 (Rolland et al. 2014), which increases P53-dependent expression of pro-apoptotic genes, i.e. Bax and Puma (Rada et al. 2017). EHMT2 is also a known interactor of EP300/CBP (Rada et al. 2017), which in turn is a known E6 binder (Supplemental Table 1). As expected, Reactome pathways were related to IFN signaling and antiviral mechanisms (Supplemental Table 6). These are again shared by AAE6 and E6AP but with different interactors (Supplemental Table 6).

Interactors common to AAE6 and EPE6

NOG2 a known interactor of IN80B (Cloutier et al. 2017) and MYC (Kalkat et al. 2018) is also involved in P53 stabilization potentially stimulating apoptosis as shown in the development of zebrafish retinal cells (Paridaen et al. 2011). In contrast, NOG2 is overexpressed in some cancers such as breast or colorectal where it stimulates G_1/S phase transition by altering P53 and cyclin dependent kinase inhibitor $p21^{Cip-1/Waf1}$ pathways (Datta et al. 2015). An interactome study indicated that in addition to IN80B, NOG2 also interacts with two other proteins selected by the peptide method, i.e. ribosome biogenesis protein NSA2 homolog (NSA2) (Huttlin et al. 2015) and

G patch domain-containing protein 4 (GPTC4) (Huttlin et al. 2017) (Supplemental Table 4). Unfortunately, these data originate from large interactome studies and the consequences of each interaction was not investigated (Huttlin et al. 2015, 2017). Therefore, the exact consequences of NOG2 interaction with NSA2 or GPTC4 remain to be determined but they could have been co-immunoprecipitated in a single complex. Surprisingly, no Reactome pathways could be aligned with NOG2 (Supplemental Table 6).

TRIPC is an E3 ubiquitin-protein ligase which shares similarities with UBE3A (E6AP). It contains the conserved homologues to E6AP carboxy terminus (HECT) domain and multiple LxxLL (where x denotes any amino acid) motifs that correspond to the E6 binding site on E6AP (Vande Pol and Klingelhutz 2013, Zanier et al. 2013, Larrieu et al. 2020). There are 4 motifs (LQALL AA position 402; LITLL AA position 485; LHFLL AA position 697; and LDQLL AA position 1862) present throughout TRIPC that could potentially allow interaction with E6. TRIPC triggers the ubiquitination and degradation of several proteins including the P53 activator protein ADP-ribosylation factor (ARF, p14 in humans) (Haupt et al. 1997, Collado and Serrano 2010) potentially doubling the effect of P53 inactivation by E6 as ARF acts upstream of E6AP-mediated P53 degradation. Interestingly, TRIPC-dependant ARF degradation is inactivated by MYC or Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) binding to TRIPC (Chen et al. 2010, Chio et al. 2012). Since MYC is a known E6 target, E6 binding to TRIPC could promote ARF degradation by stimulating TRIPC activity and E6 binding to TRIPC and/or MYC could protect TRIPC from MYC inactivation. In addition to ARF, the Brahma-related gene 1-associated factor 57 (BAF57) is another protein targeted by TRIPC. BAF57 degradation by TRIPC could be inhibited when BAF155 is bound to TRIPC (Keppler and Archer 2010). BAF57 is a canonical

component alongside BAF53 of the SWItch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex (Chen and Archer 2005)—first detected in yeast (Decristofaro et al. 2001). Interestingly, BAF53 is essential for the expression of E6 and E7 when the viral genome has been integrated into the host cell (Lee et al. 2011). TRIPC is involved in Reactome pathways mainly associated with Antigen processing and ubiquitination and Immune system (Supplemental Table 6). Interestingly, E6AP is found in all the pathways involving TRIPC and all pathways involving TRIPC are connected to AAE6 and EPE6 but with different entities (Supplemental Table 6).

CHM4B is thought to be a subunit of the endosomal sorting complex required for transport (ESCRT)-III complex involved in the formation of multi-vesicular bodies and CHM4B is important in membrane fission processes including the budding of enveloped viruses (Strack et al. 2003, Hu et al. 2015). CHM4B is overexpressed in hepatocellular carcinomas and in some head and neck squamous cell carcinomas (Gollin 2014, Hu et al. 2015). Co-IP experiments indicated that CHM4B interacts with the inhibitory P53 isoform Δ133p53α that can block the activity of full-length P53 (Horikawa et al. 2014). Other interesting interactors of CHM4B are interferon-responsive factor (IRF)-2 (Hubel et al. 2019), breast cancer-associated protein (BRCA) 2 (Malik et al. 2016) and E-cadherin (Guo et al. 2014). CHM4B presence in Reactome is mostly associated with antigen processing, HIF viral life cycle and autophagy of which some entities are shared between both variants while others differ (Supplemental Table 6): e.g. Late endosomal microautophagy pathways also include Hemoglobin subunit beta HBB (P68871) [AAE6 and EPE6], as well as HIV infection pathways also include E3 ubiquitin-protein ligase RBX1 *(*P62877), Tyrosine-protein kinase HCK (P08631) [AAE6], Cyclin-dependent kinase 9 CDK9 (P50750) and F-box/WD repeat-containing protein 1A FBW1A (Q9Y297) [EPE6].

Protein-pathway method

Data was gathered independently of treatment like done with the Peptide method above (Methodological Approach). The groups of AAE6 (n=90), EPE6 (n=61) and overlapping AAE6/EPE6 binders (n=20) selected by the Protein Method (Figure 2B; Supplemental Table 5) were each collectively analyzed using Reactome: Within the 25 most significant pathways, 26/90 entities were identified for AAE6 (Supplemental Table 7) and 48/61 for EPE6 (Supplemental Table 8). Out of 20 overlapping AAE6/EPE6 targets, 5 were identified within the 25 most significant pathways targeted by both variants whereas 8 were only seen in EPE6-targeted pathways (Supplemental Table 9). While the P-value was significant throughout for both sub-lineages and the overlapping proteins, the FDR was statistically significant ($P<0.05$) in 18/25 pathways for AAE6 only (Supplemental Tables 7, 8 and 9). Nevertheless, for comparison, results of both E6 sub-lineages and overlapping E6 targets were investigated. AAE6 and EPE6 entities belonging to these pathways are also listed. The same analysis was also done for overlapping E6 targets.

AAE6-associated pathways

AAE6 was strongly associated with 11 Notch1 signaling pathways (**1,2,3,9,10,11,12,13,15**,**16**,22) essentially with the same protein targets (Supplemental Table 7): cyclin-dependent kinase 8 (CDK8, P49336), histone acetyltransferase KAT2A (Q92830), E3 ubiquitin-protein ligase RBX1 *(*P62877) and two isoforms of the F-box/WD repeat-containing protein 7 (FBXW7, Q969H0-1, Q969H0-4). CDK8 phosphorylates the neurogenic locus notch homolog protein 1 (NOTC1) rendering it for ubiquitination and degradation through the proteasome. Consequently, NOTC1 is not acetylated by mastermind-like protein 1 (MAML1) and its transcription is not enhanced by EP300 (Popko-Scibor et al. 2011). Interestingly, it was recently

reported that “Rare driver mutations in head and neck squamous cell carcinomas converge on Notch signaling” (Loganathan et al. 2020). RBX1 and FBXW7 are also part of Wnt signaling (pathway **14**) evidencing communication between these two pathways which are deregulated in cancer. Wnt/beta catenin signaling plays a role in development and adult homeostasis. In the latter it is mostly inactive which is controlled by several kinases such as glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), axin and adenomatous polyposis coli (APC), a tumour suppressor gene often mutated in colon cancer (Verheyen and Gottardi 2010 and references therein). Interestingly, APC (P25054) was found only once with just one unique peptide yet it appeared in pathway **14** targeted by AAE6. Other notable findings were AAE6 binders belonging to the Defective base excision repair (BER) associated with the human homologue of the Escherichia coli mutY gene (hMYH), i.e. two isoforms of its encoded protein adenine DNA glycosylase (MUTYH): Q9UIF7-3 and -6 (pathways **5,7**,19), as well as P53 regulates metabolic genes (pathway **8**): P53 (P04637), MTOR (P42345), thioredoxin (THIO, P10599), trinucleotide repeat-containing gene 6C protein (TNR6C, Q9HCJ0), cytochrome c oxidase subunit 6B1 (CX6B1, P14854) and 5'-AMP-activated protein kinase subunit gamma-2 (AAKG2, Q9UGJ0). MUTYH germline mutations of BER (pathways **5,7**,19) cause MUTYH-associated polyposis (MAP), a disorder similar to familial adenomatous polyposis (FAP), caused by mutations in the APC gene (Mazzei et al. 2013). P53 regulates metabolic genes also communicates with Wnt signaling via AAKG2. Hence AAE6-targeted cellular proteins derive from the axis of Wnt and Notch1 signaling as well as Wnt signaling and P53 regulates metabolic genes. The most striking candidates for this axis are RBX1, FBXW7, KAT2A and CDK8 due to their presence in 8 to 11 pathways (Supplemental Table 7).

That pathway **8** is targeted by AAE6 but not EPE6 in this study may explain our findings that AAE6 deregulates cellular metabolism through the Warburg effect (Richard et al. 2010, Cuninghame et al. 2017) and deregulation of HIF-1α levels (Cuninghame et al. 2017) further resonating with the fact that KS6A4 (Peptide method) seems to be targeted by AAE6 only. Thus, deregulated energetics and hypoxia—two hallmarks of cancer, may cause the "higher" carcinogenic ability of AAE6. While EPE6 also targets P53, CX6B1 and THIO of pathway **8**, AAE6 may still have an advantage over EPE6 due to the additional actions of MTOR, TNR6C and AAKG2. Moreover, RBX1, FBXW7 and CDK8 (Notch1 signaling pathways **1,2,3,9,10,11,12,13,15,16**), as well as MTOR (P53 and metabolic genes pathway **8**) are known HIF-1α binders (Galbraith et al. 2013, Heir et al. 2013, Perez-Perri et al. 2016) strengthening the effect of AAE6 even more. Indeed, alteration of MTOR function is known to promote HIF-1α translation (Knaup et al. 2009). HIF-1α levels are regulated by prolyl hydroxylases in that they mark HIF-1α for proteasome degradation. This process involves HIF-1α ubiquitination by the E3 ubiquitin ligase von Hippel-Lindau protein (VHL) complex, formed by VHL, elongin B and C, Cullin-2 and RBX1 (Haase et al. 2009). HIF-1α is also degraded during hypoxia after phosphorylation by glycogen synthase kinase 3β, leading to its ubiquitination by the E3 ubiquitin ligase FBXW7 (Cassavaugh et al. 2011). HIF-1α controls the expression of hypoxia-inducible gene (HIG). CDK8 and its associated protein Mediator of RNA polymerase II transcription subunit 1 and 26 are recruited by the HIF-1α transactivation domain at the promoter of several HIGs leading to the activation of the super elongation complex (SEC) to promote RNA polymerase II (RNAPII) elongation (Galbraith et al. 2013). In addition, proto-oncogene c-Fos-encoded protein (FOS, P01100) (pathways 21,24) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (F263, Q16875), were found to be

associated with HIF-1α despite not directly interacting with it (Onnis et al. 2013, Kumar et al. 2015). HIF-1α and FOS both bind the vascular endothelial growth factor promoter under hypoxic conditions (Onnis et al. 2013). F263, a master regulator of glycolysis, is activated by HIF-1α and could be of importance for Warburg effect development (Minchenko et al. 2002).

Defective base excision repair (BER) associated with MUTYH (pathways **5,** 19) is targeted by AAE6 substantiating our finding that the AA (D2/D3) sub-lineage seems to integrate earlier into the host genome than EP (A1) as evidenced by an organotypic cell culture model of early cervical carcinogenesis (Jackson et al. 2014, 2016). Indeed, another group reported that BER is essential for the HIV provirus DNA to integrate into the host genome proposing an interesting analogy with transposable elements (Yoder et al. 2011). In "The landscape of viral association with human cancers" study led by the Pancancer Analysis of Whole Genomes Consortium (Zapatka et al. 2020), HPV16 integration seems to be associated with fragile sites or regions with limited access to DNA repair complexes. In addition, ATP-dependent chromosome remodeling complex INO80 implicated in DNA repair enhances BER activity (Hinz and Czaja 2015), and MX2—only targeted by EPE6 cannot inhibit E6 expression after the integration of its viral DNA (Pett et al. 2006), again connecting the findings between Peptide and Protein Method and underlining our earlier findings (Jackson et al. 2014, 2016). Finally, Forkhead box protein O1 (FOXO)-mediated transcription (pathway 25) overlaps between the two variants via nuclear transcription factor Y subunit alpha NFYA (P23511) (Supplemental Table 9). FOXO transcription factors act in pathways controlling cell survival, growth, differentiation and metabolism in various scenarios, such as growth factor deprivation, starvation and oxidative stress (Eijkelenboom and Burgering 2013).

EPE6-associated pathways

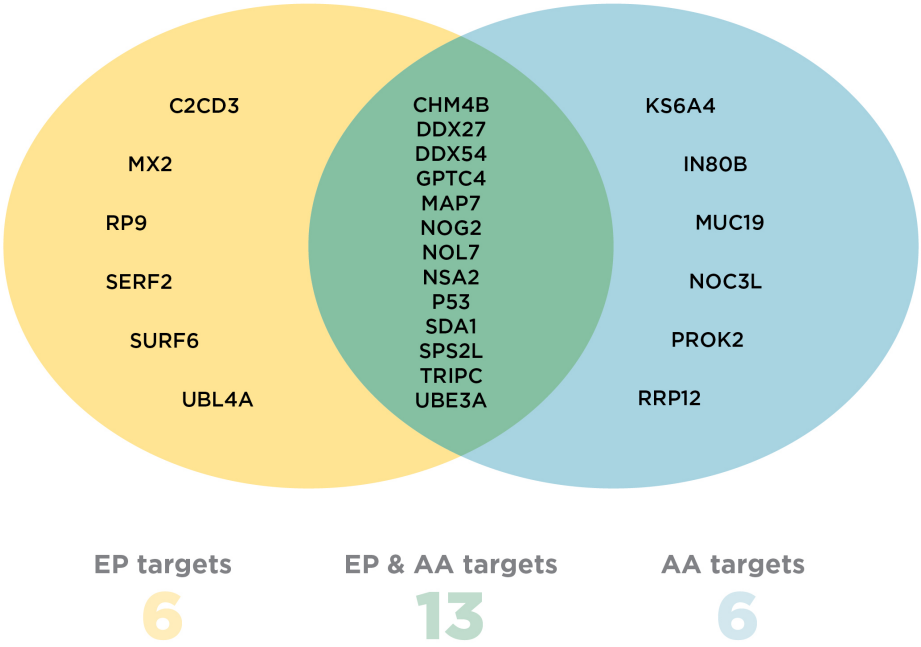
Up to 8 EPE6 interactors are part of homology directed repair (HDR) DNA damage pathways 5,9,19,22: P53, THIO, proliferating cell nuclear antigen (PCNA, P12004), CX6B1, double-strand break repair protein MRE11 (P49959), cyclin-dependent kinase 9 (CDK9, P50750), geranylgeranyl transferase type-2 subunit alpha (PGTA, Q92696) and DNA endonuclease RBBP8 (Q99708). PCNA also interacts with the adenine DNA glycosylase MUTYH (Parker et al. 2001) again linking the two E6 variants under study. However, while some of the targeted pathways overlap, most of the entities associated with the individual E6 variant differ (Supplemental Table 6). Like AAE6, EPE6 seems to target various pathways crucial for embryogenesis but deregulated in cancer such as Hippo (a major growth control pathway), PI3K/AKT, growth factor initiated Mesenchymal-epithelial signal transduction (MET) and epithelial growth factor Receptor (EGFR) (pathways 1,3,7,10,14,24). Hippo communicates with the Wnt pathway through segment polarity protein dishevelled homologue DVL-2 (DVL2, O14641) via EPE6's pathway 1 and AAE6's pathways **14** and **17**. The most notable EPE6 target seems to be PI3K regulatory subunit alpha (P85A, P27986) detected in 5 pathways related to PI3K, MET and EGFR (3,7,10,14,24), which all communicate with each other (Supplemental Table 8). Interestingly, some HIF-1α binders were also found to be targeted with EPE6, i.e. WW domain-containing transcription regulator protein 1 (WWTR1, Q9GZV5) (pathway 1) and CDK9 (pathway 22) (Galbraith et al. 2013, Maroni et al. 2015). WWTR1, also called transcriptional co-activator with PDZ-binding motif, is a component of the HIF-1α transcriptional complex and a regulator of the Hippo pathway which are both involved in increasing expression of E-cadherin during hypoxia (Maroni et al. 2015). Since CDK9 is a component of SEC recruited by CDK8 upon interaction with HIF-1α (Galbraith et al. 2013),

both AAE6 and EPE6 could potentially alter RNAPII elongation. Nevertheless, only CDK8 is essential to the process (Galbraith et al. 2013) suggesting different capabilities by AAE6 and EPE6 at modifying RNAPII elongation. Reverse co-immunoprecipitation experiments with selected candidates are warranted to confirm the above findings to establish any differential binding partners for both sub-lineages.

Pathways common to AAE6 and EPE6

The 25 most significant pathways generated for the 20 overlapping interactors of AAE6 and EPE6 are listed in Supplemental Table 9. No differential pathway enrichment was noted as all 124 pathways showed the same FDR value of 0.09. Out of 20 overlapping proteins, 15 were distributed among the top 25 pathways with 13 also found in the pathways described for AAE6 and EPE6 (P53, NFYA, DVL2, THIO, CX6B1, CHM4B, HBB, inactive polyglycylase tubulin--tyrosine ligase-like protein 10 (TTL10, Q6ZVT0), tubulin monoglycylase TTLL3 (Q9Y4R7), SH3 domain-containing kinase-binding protein 1 (SH3K1, Q96B97), fibrinogen alpha chain (FIBA, P02671), coiled-coil domain-containing protein 59 (TAP26, Q9P031) and testisin PRSS21 (Q9Y6M0)). Consequently, most pathways identified with these proteins were involved in the same pathways as those described for AAE6 or EPE6 above, albeit with fewer entities (Supplemental Table 9). These results suggest that several pathways are targeted by both AAE6 and EPE6, but with different efficiencies (Supplemental Table 9). The two remaining proteins, protein lin-37 homologue (Q96GY3) and centromere protein R (Q13352) were found in pathway 14 (Cell Cycle, Mitotic) alongside P53 and CHM4B. Overall, P53 is the main contributor found in 13 of the 25 pathways (1,4,7,10,12,13,14,18,19,20,23,24; Table 4), and is the only entity found in 9 of them. Interestingly, P53 appears to be the most important target between AAE6 and EPE6.

A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NSA2
P53
SDA1
SPS2L
TRIPC
UBE3A
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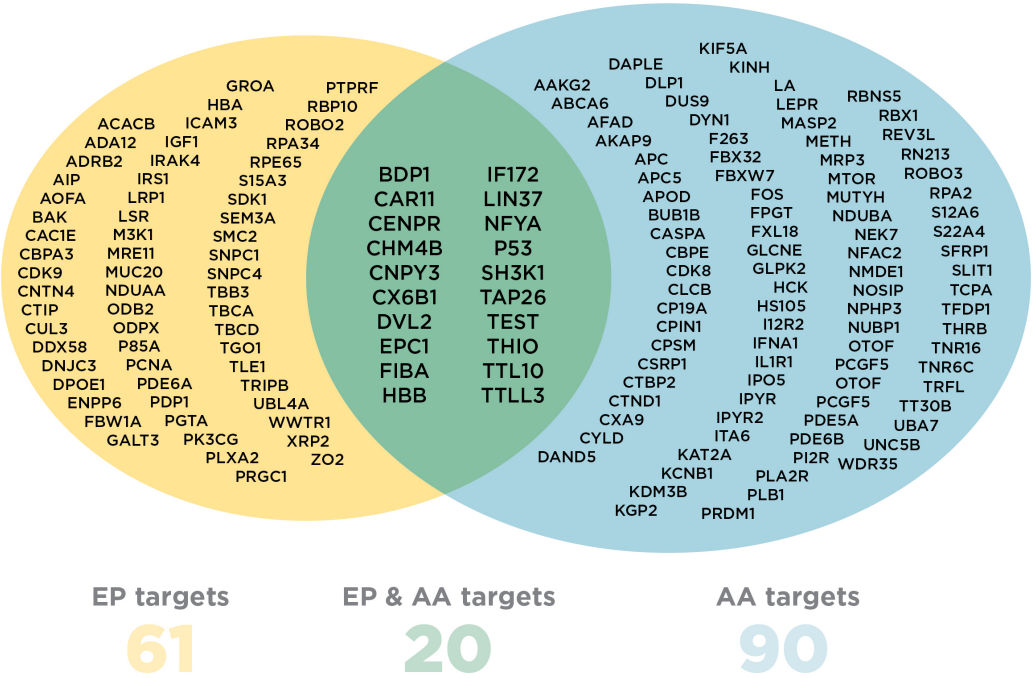
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IGF1
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ADRB2
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IRS1
S15A3
AOFA
LRP1
SDK1
BAK
LSR
SEM3A
CAC1E
M3K1
SMC2
CBPA3
MRE11
SNPC1
CDK9
MUC20
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CTIP
ODB2
TBCA
CUL3
ODPX
TBCD
DDX58
P85A
TGO1
DNJC3
PCNA
TLE1
DPOE1
PDE6A
TRIPB
ENPP6
PDP1
UBL4A
FBW1A
PGTA
WWTR1
GALT3
PK3CG
XRP2
PLXA2
ZO2
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CNPY3
CX6B1
DVL2
EPC1
FIBA
HBB
IF172
LIN37
NFYA
P53
SH3K1
TAP26
TEST
THIO
TTL10
TTLL3
KIF5A
DAPLE
KINH
AAKG2
DLP1
LA
ABCA6
DUS9
LEPR
RBNS5
AFAD
DYN1
MASP2
RBX1
AKAP9
F263
METH
REV3L
APC
FBX32
MRP3
RN213
APC5
FBXW7
MTOR
ROBO3
APOD
FOS
MUTYH
RPA2
BUB1B
FPGT
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S12A6
CASPA
FXL18
NEK7
S22A4
CBPE
GLCNE
NFAC2
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HCK
NOSIP
TCPA
CP19A
HS105
NPHP3
TFDP1
CPIN1
I12R2
NUBP1
THRB
CPSM
IFNA1
OTOF
TNR16
CSRP1
IL1R1
PCGF5
TNR6C
CTBP2
IPO5
OTOF
TRFL
CTND1
IPYR
PCGF5
TT30B
CXA9
IPYR2
PDE5A
UBA7
CYLD
ITA6
PDE6B
UNC5B
DAND5
KAT2A
PI2R
WDR35
KCNB1
PLA2R
KDM3B
PLB1
KGP2
PRDM1
EP targets
61
EP & AA targets
20
AA targets
90

Figure 2 — Venn diagrams representing AAE6 and EPE6 host cellular targets for both selection approaches: Peptide method (A) and Protein-pathway method (B). EPE6-specific interactors are in yellow, AAE6-specific interactors are in blue and proteins common to both variants are in green.

4. Conclusions

Functional effects driven by AAE6 and EPE6 showed differences and similarities in this study (Figure 3). While further experiments are warranted some conclusions may be drawn. Earlier findings were consolidated by present findings bridging them by our group. Using two different approaches to dissect both variants it has become increasingly clear that AAE6 seems to take the lead in targeting hypoxia and energetics pathways, and that both E6 proteins can equally inactivate p53 by multiple means. The latter along with the fact that AAE6 is also very active in subverting Notch1 signaling are completely new discoveries and are strengthened by the finding that Notch1 signaling is required to maintain the undifferentiated cell state in various stem and precursor cell populations (Gustafsson et al. 2005). Likewise, the identification of MX2 which on the one hand is associated with EPE6 but on the other may hamper integration is an interesting addition to the notion that AAE6 may be more successful in viral integration than EPE6. We are nevertheless mindful that our findings must be complemented with additional wet lab experiments such as reverse co-immunoprecipitation and RNA interference to validate our current findings and to elucidate gain or loss of function of reported candidate binders.

Next steps will be to reconcile our previous work with data reported in this interactome study, i.e. confirming the interaction between E6 variants and KS6A4, three P53 regulating proteins (NOG2, TRIPC, and CHM4B) and E6's role with NOTCH signaling, hypoxia and defective base excision repair (Figure 3). Although these are a small subset of identified proteins,

confirming their interactions will provide vital information about E6's ability to target P53 redundantly, and increase hypoxia via altering the Warburg Effect. These are two cancer hallmarks that, if differentially affected by E6 variant proteins, can prove useful when developing therapeutic agents against HPV infections.

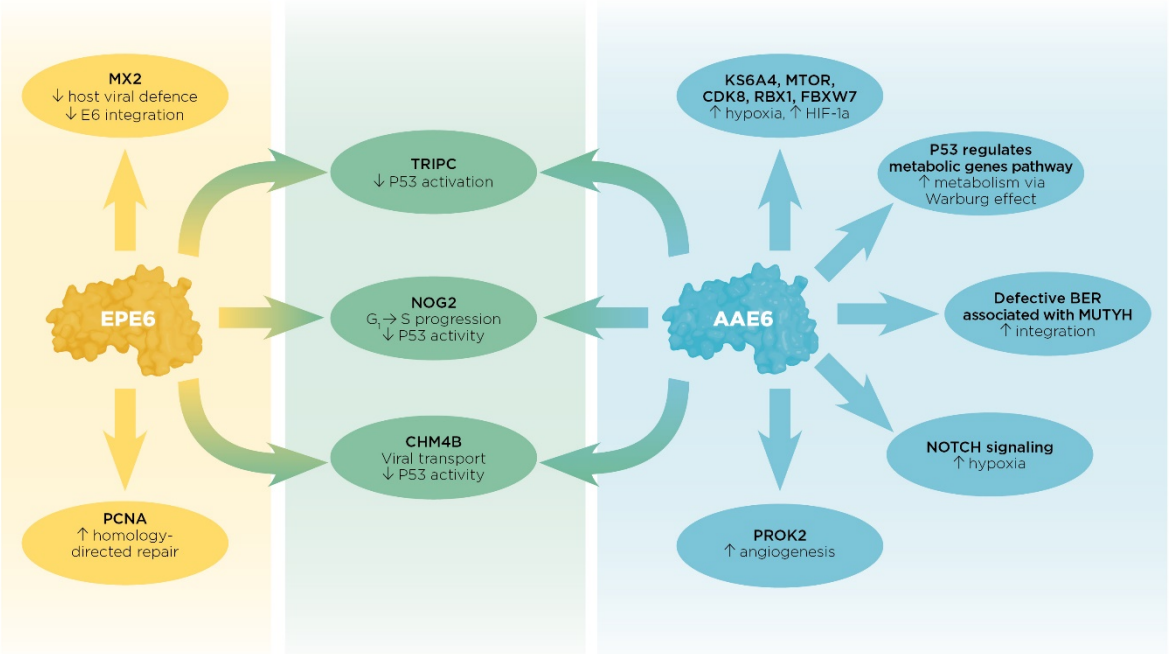

Figure 3 – Individual and tandem effects between AAE6 and EPE6 interactions with host cellular proteins and pathways by which E6 variants may promote carcinogenesis. Deductions were made by interlinking Peptide and Protein-pathway method. We show that the first-described E6 binder (P53) is most likely targeted through several mechanisms shared by both variants (middle). AAE6 and EPE6 proteins equally targeted three proteins known to affect P53 inactivation: TRIPC, CHM4B, and NOG2. TRIPC in the presence of E6 can result in increased P53 activator protein inhibition, thus inhibiting P53 activity itself. E6 can also destabilize and degrade MYC, which inhibits TRIPC ubiquitination, again causing inhibition of P53 activation. NOG2—altering P53 and cyclin dependent kinase inhibitor p21Cip-1/Waf1 pathways is another potential mechanism for how both E6 variant proteins could target P53. Finally, AAE6's interaction with CHM4B may further inhibit full-length P53 as CHM4B is known to interact with Δ133p53α, an inhibitory isoform of P53. Potential interactions unique to AAE6 are heavily associated with hypoxia and HIF-1α. AAE6 (right) interacts with KS6A4, MTOR, RBX1, CDK8, and FBXW7, likely resulting in increased activity/transcription of HIF-1a, increasing hypoxia. In addition, RBX1, CDK8 and FBXW7 are also

involved in NOTCH signaling potentially dysregulating metabolism. The interaction between AAE6 and the P53 regulates metabolic genes pathway likely results in altered metabolism by the Warburg effect. AAE6 binding to PROK2 could promote angiogenesis within infected cells. Finally, AAE6 likely possesses increased genomic integration capabilities as it likely affects the Defective BER associated with MUTYH pathway and potentially degrades or inhibits its interaction with MX2. In contrast, EPE6's binding to MX2 (left) may limit this variant's ability to integrate into the host genome effectively while it may be more successful in deregulating the host's viral defence. EPE6's targeting PCNA may interfere with BER antagonistically to AAE6 and lead to less chromosomal instability.

The process of filtering proteins poses a substantial limitation in bioinformatics as there are many ways to analyze MS datasets. Most groups filter datasets by removing proteins involved in negative controls, along with setting a minimum number of peptides required to be a valid result. The additional use of the Protein-pathway to the Peptide method provided a more inclusive way for identifying potential E6 interacting proteins and their processes. When compared to earlier works that report single interactors (Supplemental Table 1), we found that several processes to those that we identified here seemed to be of importance: pathways hijacked during cancer causing their dysregulation from embryogenesis and wound healing, tumour suppressor and oncoproteins, DNA damage response and immune system. The list, however, lacked entities associated with cellular energetics and hypoxia—an important group from this present and our past studies (Richard et al. 2010, Cuninghame et al. 2017). Even though AAE6 and EPE6 are sometimes associated with the same Reactome pathways, the identified entities differ, which may result in different net function outcomes. This and the potentially different binding affinities discovered here may explain some of the underlying mechanisms in altered carcinogenesis. The current study is one of few reporting PPIs in a global perspective focusing on common E6 sub-lineages with varying prevalence in cervical cancer.

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6. Conflict of interest
We have no conflict of interest to declare.

7. Author contributions
M.T. and I.Z. conceived the idea of the project and contributed to the initial experimental design, which was summarized in a report by M.T. M.M. did all the wet lab work supervised by G.D. and both largely extended and managed experimental design. M.M. and I.Z. corresponded with the MS lab. G.D. and I.Z. performed extensive data analyses with input from M.M. and managed heatmaps and Reactome tables. I.Z. drafted the manuscript with substantial input from G.D. and critical feedback from M.T. and M.M. M.M. created Supplemental Table 1 with revisions by I.Z. as well as all Figures with revisions by I.Z. and G.D. All authors have read and approved the final manuscript.

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