The APOBEC3 genes and their role in cancer: insights from human papillomavirus

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Abstract

The interaction between human papillomaviruses (HPV) and the apolipoprotein-B mRNA editing catalytic polypeptide-like (APOBEC)3 (A3) genes has garnered increasing attention in recent years, with considerable efforts focused on understanding their apparent roles in both viral editing and in HPV-driven carcinogenesis. Here we review these developments and highlight several outstanding questions in the field. We consider whether editing of the virus and mutagenesis of the host are linked, or whether both are essentially separate events, coincidentally mediated by a common, or distinct A3 enzymes. We discuss the viral mechanisms and cellular signalling pathways implicated in A3 induction in virally-infected cells, examine which of the A3 enzymes might play the major role in HPV-associated carcinogenesis and in the development of therapeutic resistance. We consider the parallels between A3 induction in HPV-infected cells and what might be causing aberrant A3 activity in HPV-independent cancers such as those arising in the bladder, lung and breast. Finally, we discuss the implications of ongoing A3 activity in tumours under treatment and the therapeutic opportunities that this may present.

Introduction

A link between sexual contact and cervical cancer was first reported in 1842, by the Italian physician, Rigoni-Stern (Rigoni-Stern 1842), yet the role of human papillomaviruses (HPVs) as the incriminating infectious agent was not substantiated until the 1970s; at which time, intranuclear papillomavirus particles were found within koilocytic epithelial cells of cervical

condylomatosis (Torre *et al.* 1978; Hills & Laverty 1979). By 1983, the DNA of HPV-16 and HPV-18 had been successfully isolated from cervical cancer biopsies (Dürst *et al.* 1983; Boshart *et al.* 1984) but the many molecular mechanisms by which these viruses cause cancer continue to be elucidated.

HPVs are small, non-enveloped DNA viruses, consisting of an 8kb circular genome (Figure 1A) encased in a viral capsid. There are over 200 different genotypes with tropisms for stem cells in the basal layer of either cutaneous or mucosal epithelia, in which the viral life cycle is tightly linked to and dependent upon keratinocyte differentiation (Figure 1B). The HPV genome consists of six early genes responsible for viral genome maintenance and amplification and two late genes (L1 and L2) which encode the viral capsid proteins and are expressed in terminally differentiated keratinocytes immediately prior to host cell death and release of virions (for detailed reviews see Doorbar *et al.*, 2015; McBride, 2017). Infection is typically either asymptomatic, or associated with benign warts. At least 14 HPV types however (including HPV-16 and HPV-18), are carcinogenic, and these 'high-risk' (HR-HPV) types cause human cancers in the mucosal epithelia of several sites, including the cervix, vulva, vagina, penis, anus, and oropharynx (tonsils and tongue base). The vast majority of HR-HPV infections are cleared naturally within 12-18 months by the host immune system (Richardson *et al.* 2003; Bodily & Laimins 2011), yet globally, HPV infection accounts for over 600,000 cancers (90% of which are cervical cancers) and 250,000 deaths per year (de Martel *et al.* 2017). The complex biology underlying HPV-associated carcinogenesis is the subject of many detailed reviews (e.g. Bodily & Laimins 2011; Doorbar *et al.* 2015; Lechner & Fenton 2016). Here we will focus on the emerging role that one or more of the apolipoprotein-B mRNA editing catalytic polypeptide-like-3 (APOBEC3 or A3) family of innate immune response genes appear to play in this process, including the generation of somatic alterations to the host genome that in addition to viral oncoprotein expression, are required for HPV-associated carcinogenesis.

Current model of HPV-induced carcinogenesis

Papillomaviruses rely on host DNA polymerases and the DNA damage response for replication and amplification of their genomes, and must therefore induce cell cycle entry upon infection; a process which is driven by two viral early genes, E6 and E7 (reviewed in Doorbar *et al.*, 2015; McBride, 2017). E6 and E7 from high-risk HPV types (HR-E6/HR-E7, also known as the HPV oncogenes) harbour several activities not shared by their low-risk homologues, which appear to be important for carcinogenesis and may serve to trigger the mutagenic activity of APOBEC3 (A3) proteins seen in HPV-associated cancers. In particular, the induction of replication stress, host DNA repair responses and downregulation of the pRB and p53 tumour suppressors (Munger & Jones 2015) are key activities of HR-E6 and E7 that will be discussed in this context. During productive infection, the expression of E6 and E7 is restricted to the basal and parabasal layers of the epithelium and at later stages is repressed by the viral E2 protein but in a small fraction of HR-HPV infections the virus persists and cells with increased E6/E7 expression gain a selective growth advantage, populating the upper epithelial layers. Differentiation of these cells is blocked, resulting in loss of additional viral gene expression and exit from the productive life cycle. In the cervix this can be observed in the transition from early cervical intraepithelial neoplasia (CIN1), to precancerous CIN2/3 lesions that forms the basis of cervical cancer screening. In CIN3 lesions and invasive carcinoma, integration of the virus into the host genome and loss of viral episomes is commonly observed, with a selection *in vivo* for clones in which integration has disrupted the E2 gene, permitting further increases in E6/E7 expression (Bodily & Laimins 2011; Doorbar *et al.* 2015).

The A3 genes and somatic mutagenesis in cancer

The rate at which somatic mutations accumulate in cells is governed both by the rate at which DNA damage occurs and by the fidelity with which it is repaired or damaged cells are eliminated by apoptosis. Loss of p53 could explain persistence of cells carrying DNA damage but which mutational processes generate the mutations in HPV infected cells and does HPV increase the rate at which DNA damage occurs? The wealth of somatic mutation data generated by large-scale cancer genomics efforts such as The Cancer Genome Atlas (TCGA) project and the International Cancer Genome Consortium (ICGC) has recently enabled identification of mutational signatures - distinctive patterns that can reveal the mutational processes operational

in different tumours (Nik-Zainal *et al.*, 2012; Alexandrov *et al.*, 2013a; Alexandrov *et al.*, 2013b). As might be expected, skin cancer genomes are dominated by CC>TT mutations consistent with those caused by ultraviolet light in experimental systems, while cancers associated with tobacco smoking display a mutational signature (G>T and GG>TT mutations) implicating tobacco carcinogens such as benzo(a)pyrene. In both cases, the mutation signatures display a transcriptional strand bias that is consistent with the known role for transcription-coupled nucleotide excision repair in resolving such lesions (Alexandrov *et al.*, 2013). Other mutational signatures arise from specific defects in the pathways responsible for repairing DNA damage; at least four signatures have been linked to defects in mismatch repair for instance, while defects in double strand break repair by homologous recombination give rise to a signature observed in tumours harbouring *BRCA1* or *BRCA2* mutations (https://cancer.sanger.ac.uk/cosmic/signatures, Forbes *et al.*, 2017). The mutational signatures observed in tumour samples (or indeed in healthy tissue) are therefore shaped both by the processes that have caused damage to the DNA during the lifetime of the individual and by the pathways (or defects therein) responsible for repairing that damage.

Strikingly, cancers in several tissues, including breast, lung, bladder, cervix and head and neck frequently display two closely-related signatures characterized by C>T transitions and C>G transversions at TpC dinucleotides that have been attributed to the deoxycytidine deamination activity of one or more APOBEC enzymes (Burns *et al.*, 2013a; Burns *et al*, 2013b; Alexandrov *et al.*, 2013a; Roberts *et al.*, 2013; Taylor *et al.*, 2013). Humans possess 11 APOBEC genes, with physiological roles including antibody diversification (Activation-Induced Cytidine Deaminase, *AICDA*), cellular mRNA editing (*APOBEC1*) and inhibition of exogenous virus and endogenous retroelement replication, which are mediated by members of the 7-gene APOBEC3 (A3) family, (Figure 2), reviewed in (Holmes *et al.* 2007; Conticello 2008; Harris & Dudley 2015). Soon after the cloning of *APOBEC1*, it was shown that liver-specific overexpression in transgenic mice or rabbits caused hepatocellular carcinoma (Yamanaka *et al.* 1995). The subsequent demonstration that APOBEC1, several A3 enzymes and AICDA (AID) could deaminate single-stranded (ss)DNA in addition to RNA (Harris *et al.* 2002; Petersen-Mahrt *et al.* 2002), together with the finding that transgenic *AICDA* mice were also cancer-prone (Okazaki *et al.* 2003) suggested a potential role for mutagenic APOBEC/AID

activity in the development of human cancers; a hypothesis that awaited large-scale testing until the advent of next-generation sequencing (NGS) and the detection of the aforementioned mutational signatures in tumour exomes. APOBEC1 and several of the closely-related A3 enzymes (A3A, B, C, D, F and H) display a preference for deamination of TpC sites in ssDNA *in vitro* that is consistent with the TpC mutational signatures observed in cancer genomes, with gene expression analysis and loss-of-function experiments in breast cancer cell lines suggesting a prominent role for A3B (Burns *et al.*, 2013a). Distinct A3G and AID mutational signatures have also been detected across a wide range of cancer types (Rogozin *et al.* 2019) but for the purposes of this review we focus on the TpC signatures, henceforth referred to as APOBEC-associated. Analyses of cancer genome sequencing data and studies in cells overexpressing A3A or A3B suggest the major exposure of ssDNA substrate for A3 activity in tumour cells arises on the lagging strand during DNA replication, presumably as a result of replication fork stalling due to replication stress (Green *et al.* 2016; Haradhvala *et al.* 2016; Hoopes *et al.* 2016; Morganella *et al.* 2016; Seplyarskiy *et al.* 2016). Unlike other mutational signatures, A3-mediated mutations are frequently enriched in early-replicating regions of the genome, although interestingly this effect is more pronounced in lung and bladder cancer exomes from TCGA than in cervix and is not apparent in head and neck squamous cell carcinoma (HNSCC) (Kazanov *et al.* 2015).

The strong enrichment of the APOBEC signature in cervical cancer exomes (Burns, Temiz and Harris, 2013; Alexandrov *et al.*, 2013; Roberts *et al.*, 2013), together with previous evidence for A3 editing of human papillomavirus (HPV) genomes in plantar warts and precancerous cervical lesions (Vartanian *et al.* 2008) suggested that the presence of HPV in cells might somehow induce or potentiate A3 activity, damaging the host genome and resulting in the observed enrichment of these mutational signatures in HPV-associated cancers (Kuong & Loeb 2013). Having identified A3B among a list of genes that are consistently upregulated in HPV-associated malignancies irrespective of anatomic site, we tested for such an association in HNSCC, observing increased *APOBEC3B* expression and enrichment of the APOBEC mutational signature in the ~15% of HPV-associated cases in the CGA HNSCC cohort, the majority of which are oropharyngeal tumours. We also noted a distinctive pattern of APOBEC signature mutations in exon 9 of the *PIK3CA* proto-oncogene in HPV+ HNSCC and in other

cancer types displaying the APOBEC mutational signature, thus directly implicating APOBEC activity in the generation of oncogenic driver events (Henderson *et al.* 2014; Chakravarthy *et al.* 2016). These findings were subsequently confirmed by TCGA (The Cancer Genome Atlas Network 2015) and by recent analyses of expanded (Gillison *et al.* 2019) and independent (Qin *et al.* 2018) HPV+ HNSCC cohorts . In a separate study published the same year, Vieira and colleagues also reported the enrichment of APOBEC signature mutations in HPV+ CGA HNSCCs and showed induction of A3B mRNA expression and deaminase activity in keratinocytes by E6 from the two major high-risk HPV types, HPV16 and HPV18 (Vieira *et al.* 2014). Consistent with these observations, APOBEC signature mutations are also enriched in HPV+ penile carcinoma exomes, with those tumours harbouring higher viral loads displaying greater enrichment (Feber *et al.* 2016). In further work, Pyeon and colleagues noted upregulation of both A3A and A3B expression in precancerous cervical lesions and demonstrated their induction by E7 in keratinocytes (Warren *et al.* 2015a). The same group have since shown that E7 from HR-HPV types can stabilize A3A protein by blocking its polyubiquitination by cullin-RING-based E3 ubiquitin ligase complexes (Figure 3), thus HPVs appear to modulate A3 expression at multiple levels (Westrich *et al.* 2018). Also of note are roles that A3 enzymes may play in HPV-associated cancer that are independent of their mutagenic activity against the host genome. Intriguingly, Periyasamy and colleagues have shown that A3B associates with the oestrogen receptor (ER) in breast cancer cell lines and co-activates ER target genes (Periyasamy *et al.* 2015a). The proposed mechanism involves deamination of promoter sites by A3B, leading to recruitment of DNA repair proteins and local chromatin remodelling. The cervical epithelium is also an oestrogen-responsive tissue; indeed HPV E6/E7-driven cervical cancer development in transgenic mice can be promoted by oestradiol infusion over several months (Brake & Lambert 2005). It is possible then, that A3B could also fuel cervical carcinogenesis via this non-mutagenic but nonetheless deaminase-dependent transcriptional activity.

A3 genes and viral restriction

Numerous studies indicate an important role for A3 genes in innate immunity and it is presumably an aberrant triggering and/or regulation of this response that results in the somatic

mutagenesis observed in cancer. Coincident with the cloning of the human A3 genes (Jarmuz *et al.* 2002) and the discovery that they can deaminate ssDNA (Harris *et al.* 2002), a series of seminal papers demonstrated a role for A3G (originally termed CEM-15) in HIV-1 restriction (Sheehy *et al.* 2002) and revealed a deaminase-dependent mechanism involving extensive editing of the first strand cDNA and resulting in G-to-A mutations on the positive strand (Harris *et al.* 2003; Mangeat *et al.* 2003; Zhang *et al.* 2003), although APOBEC3G also exerts deaminase-independent antiviral activity against HIV-1 (Newman *et al.* 2005). Other A3 enzymes, notably A3F and A3DE, also appear to function in HIV-1 restriction in lymphocytes, while A3A is required in monocytes – a cell type in which it is highly expressed. Unlike A3G however, it is not incorporated into HIV virions and may act together with A3G in this capacity (reviewed in (Chiu & Greene 2008).

One major obstacle to the study of A3 function *in vivo* is the greatly increased complexity of the A3 locus in primates compared with model organisms. Rodents possess only one A3 gene: a double-domain enzyme most closely related to A3G (Conticello *et al.* 2005), thus dissecting the roles of individual A3 genes in an organismal context remains a challenge. Use of murine A3 (mA3) knockout mice (which are viable and fertile) has clearly demonstrated that it functions as a cell-autonomous restriction factor for exogenous murine retroviruses including mouse mammary tumour Virus (MMTV), Friend murine leukaemia virus (MLV) and to a lesser extent, Moloney murine leukaemia virus (MoMLV), with recent work suggesting a primarily deaminase-independent mechanism (Okeoma *et al.* 2007, 2009; Stavrou *et al.* 2018).

Several A3s including A3A also inhibit Long Interspersed Element-1 (LINE-1) retrotransposition, through a mechanism that appears to involve deamination of single-stranded cDNA exposed by the action of RNase-H upon RNA/DNA hybrids (Richardson *et al.* 2014). Indeed, it appears likely that the activity against endogenous retroviruses drove the expansion of the A3 family seen in primates and other mammals, since it predates the appearance of lentiviruses (Conticello *et al.* 2005; Chiu & Greene 2008). It was recently proposed that this activity against retroelements could ameliorate the loss of LINE1 silencing caused by E7 inhibition of RB1, thus providing a potential explanation for why HPV causes A3 upregulation (Wallace & Münger 2018).

An activity against DNA viruses was first shown for A3A, in studies demonstrating inhibition of adeno-associated virus replication through a deaminase-independent mechanism (Chen *et al.* 2006; Narvaiza *et al.* 2009). These *in vitro* experiments were supported by a study in which a human A3A transgene (but not A3G) expressed in the mA3 knockout background reduced infectivity of a murine parvovirus without evidence of viral genome editing, while neither A3A nor A3G inhibited herpesvirus infection in this *in vivo* model (Nakaya *et al.* 2016). HPV pseudovirions produced in 293T cells overexpressing A3A or A3C displayed decreased infectivity, while A3A knockdown increased infectivity, suggesting these A3s may act as HPV restriction factors *in vivo* (Ahasan *et al.* 2015; Warren *et al.* 2015a) but the mechanism by which A3A inhibits HPV awaits full elucidation. Although the deaminase activity appears to be required, evidence of editing was not detected in HPV pseudovirion genomes from cells over expressing A3A, leading to the suggestion that its recently described RNA-editing activity may be responsible (Sharma *et al.* 2015; Warren *et al.* 2017). On the other hand, HPVs are subject to A3 editing *in vivo*, as first reported by Vartanian and colleagues (Vartanian *et al.* 2008), see below for detailed discussion. It appears that A3A and A3C may act on HPV at different levels, as cells expressing A3A contained reduced levels of encapsidated pseudovirions, while A3C was found to physically interact with the L1 viral capsid protein, potentially inhibiting infectivity by interfering with viral entry into target cells (Ahasan *et al.* 2015). Of note, several groups have reported cell cycle arrest upon transfection of APOBEC3A and have linked this to DNA damage caused by its deaminase activity against genomic DNA (Landry *et al.* 2011; Land *et al.* 2013; Mussil *et al.* 2013). Since HPV replication is dependent upon host cell transit through S-phase, it will be interesting to determine whether the restriction activity observed *in vitro* is due to a direct effect on the virus, or whether it is an indirect consequence of an A3A-mediated cell cycle arrest.

A3s as HPV editors?

As discussed above, transient transfection experiments using HPV pseudovirions in 293FT cells suggested possible roles for A3A and A3C in HPV restriction but did not implicate viral genome editing in this process. In W12, an HPV-16+ cell line originally derived from a low-grade CIN lesion (Stanley *et al.* 1989), over-expression A3A or A3G did not reduce virus copy number but did result in editing of the *E2* gene, as detected by the highly-sensitive 3D-PCR method originally used to demonstrate editing of the HPV-1a and HPV-16 LCRs in warts and

precancerous cervical lesions respectively (Vartanian *et al.* 2008; Wang *et al.* 2014). Editing was likewise detected upon treatment of W12 cells with IFN- β, which induced expression of A3A, A3F and A3G (Wang *et al.* 2014). Editing of the HPV-16 *E2* gene in precancerous cervical lesions has also been demonstrated using 3D-PCR (Kukimoto *et al.* 2015) and these observations have since been supported by NGS of the entire HPV-16 genome, revealing the expected strand-coordinated C:G>G:A transitions overrepresented at TpC sites throughout the early genes but enrichment within the LCR (Wakae *et al.* 2015). The authors speculate that enrichment for A3 editing in the LCR could result from increased exposure of single-stranded DNA at the origin of replication and/or transcription from the p97 promoter both located in this region (Figure 1). This study also reported A3A and A3C to be the most abundant A3 transcripts in the one HPV-infected cervix examined, while A3B was expressed at much lower levels. It should be noted that the frequency of HPV editing detected in all these studies was significantly lower than that detected for other viruses known to be edited by A3s, such as HIV-1 or HBV (Wakae *et al.* 2015). Indeed, in the W12 cell system it was necessary to block repair of deaminated cytosines with an inhibitor of uracil-DNA glycosylase to reveal editing, even when using highly sensitive techniques such as 3D-PCR or NGS for detection (Wang *et al.* 2014).

Taken together, these studies suggest that if A3s are playing a role in HPV restriction *in vivo*, it is likely to be either much less effective than the response against viruses such as HIV-1, or that is proceeds via an editing-independent mechanism, as suggested by the pseudovirion studies (Ahasan *et al.* 2015; Warren *et al.* 2015a). Nevertheless, low-level HPV editing by A3s could still contribute to HPV pathology, by generating variation that could facilitate evasion of host adaptive immune responses, analogous to the role that sublethal A3-mediated editing appears to play in HIV-1 immune escape (reviewed in Venkatesan *et al.*, 2018).

Papillomaviruses hijack the host DNA repair machinery for the amplification stage of their replication cycle, specifically homologous recombination (recombination-dependent replication (RDR)), which allows very high fidelity viral replication consistent with the very slow rate of papillomavirus evolution; approximately $2x10^{-8}$ nucleotide substitutions per site per year in the coding region (Rector *et al.* 2007; Sakakibara *et al.* 2013). Thus unlike RNA viruses, in which low-fidelity replication generates considerable variation, editing, even at a low frequency likely represents an important source of papillomavirus variation. RDR occurs independently of host DNA replication, in an extended G2-like cell cycle phase that the virus

maintains in differentiating keratinocytes and although this results in very high fidelity replication, depending on the precise mechanism it may also involve the generation of long stretches of single-stranded DNA (Sakakibara *et al.* 2013), thus potentially exposing the viral genome to A3 activity. Intriguingly, a recent analysis of cancer gene expression data has shown that A3B is co-expressed with multiple DNA damage response and G2/M-phase cell cycle genes, suggesting it might be induced in precisely this context (Ng *et al.* 2019). Indeed, a recent study in which HPV was sequenced from 124 CIN lesions and 27 invasive cervical carcinomas supports a role for A3s in generating within-host sequence diversity as assessed by looking for minor variants (allele frequency of greater than 0.5%) in NGS data, with the greatest proportion of A3 signature mutations observed in CIN1 lesions, suggesting this process is primarily acting during productive infection, when HPV is actively replicating (Hirose *et al.* 2018). As the authors of this study point out, editing of HPV at this point may be favoured by exposure of ssDNA during viral replication but would also be consistent with a role for within-host editing in generating variation prior to viral release and subsequent inter-host transmission, and therefore contributing to viral evolution. In this regard, A3 activity has been invoked as the cause of TpC dinucleotide depletion in the mucosal alpha-papillomaviruses, of which the HR-HPVs are examples (Warren *et al.* 2015b). This TpC depletion has primarily occurred at the third codon position in viral open reading frames, as might be expected given the preservation of amino acid sequence permitted, meaning the A3 editing activity observed in current HPV genomes frequently affects the first or second codon positions, resulting in non-synonymous mutations (Hirose *et al.* 2018). Although such mutations would frequently be deleterious, those that do not compromise fitness could aid evasion of host adaptive immune responses by altering viral antigens and therefore undergo positive selection, at least within-host. In tumours, the HPV sequence observed reflects not only the editing that has occurred but also the effect of selection against loss of (and possibly for enhancement of) host cell fitness. This purifying effect (along with the loss of episomal HPV DNA frequently observed upon progression) likely explains the reduced intra-sample sequence diversity observed in CIN3 and invasive lesions in this study. This observation is also consistent with the findings from a much larger-scale study in which HPV-16 genomes from 5,570 samples representing productive (largely cervical), precancerous and invasive lesions were sequenced, revealing a remarkable degree of inter-host variation that was again highest in productive lesions. In this study, the authors observed that approximately 80% of individuals harboured unique (differing by at least two nucleotides from other samples) HPV-16 genomes, with the sequence context in which these variants occurred again implicating A3 activity in HPV evolution (Mirabello *et al.* 2017). Taken together then,

A3 editing of HPV occurs at a frequency much lower than that observed for retroviruses such as HIV-1, rendering a role for deamination in HPV restriction highly unlikely. Rather, the low level of editing detected in these sequencing studies suggests an ongoing role for A3 activity in shaping HPV evolution by introducing variation otherwise lacking in a virus that is replicated with such high fidelity.

Modulation of A3 gene expression by HPV

Tight regulation of A3 expression and activity and is presumably essential for limiting their potential mutagenic activity but as we have discussed, in addition to a possible restriction activity against HPV, there may be an evolutionary advantage to the virus from inducing at least a certain level of A3 expression (see Figure 3 for a summary of pathways currently implicated in A3A and A3B transcriptional regulation and their modulation by HR-E6 and E7 proteins). Whether induction of A3 expression by HR-HPV types is a trait that the viruses have evolved to promote adaptation and immune evasion, a host response mechanism that has evolved to inhibit viral replication or a combination of the two, it may be an important cause of A3-mediated host genome mutagenesis and therefore of viral carcinogenesis. One likely candidate for host genome mutagenesis, the nuclear-localised A3B, is expressed at low basal levels in normal adult tissues but it is often highly expressed in cancer biopsies, at least at the mRNA level (Jarmuz *et al.*, 2002; Burns *et al.*, 2013a) suggesting it may be playing an important ongoing role in mutagenesis at the time of diagnosis and potentially therefore, in driving therapeutic resistance. Indeed, high A3B mRNA levels in biopsy specimens are associated with poor prognosis in oestrogen receptor (ER)+ breast cancer (Sieuwerts *et al.* 2014; Periyasamy *et al.* 2015b; Law *et al.* 2016). Unlike in breast and ovarian cancer, A3B mRNA levels are not correlated with A3 signature mutation burden in HPV-associated cancers (Roberts *et al.* 2013; Henderson *et al.* 2014; Ojesina *et al.* 2014) but A3B expression is consistently elevated in HPV-associated cancers in comparison to both normal tissue and to HPV-independent cancers arising at equivalent anatomic sites (Chakravarthy *et al.* 2016). These observations, together with the aforementioned studies demonstrating A3B upregulation by HR-HPV types (Vieira *et al.* 2014; Warren *et al.* 2015a), suggest an important role for A3B in HPV-associated cancer but also possibly in the viral life cycle. Here we review several recent studies that have detailed various mechanisms by which HPV modulates expression of A3B and other A3 genes.

Mori and colleagues identified two E6-responsive regions in the A3B promoter: basal promoter activity in human keratinocytes can be activated by E6 at a distal region (-200 to -51), while a proximal region (+1 to +45) exerts inhibition of gene expression which can be relieved by E6, acting through the zinc finger protein ZNF384 through an as-yet unknown mechanism (Mori *et al.* 2015). Consistent with previous findings from the Harris lab (Burns *et al.*, 2013a), Periyasamy *et al.* recently demonstrated an inverse relationship between *TP53* status and A3B expression levels in both primary breast tumours and breast cancer cell lines (Periyasamy *et al.* 2017). As mentioned earlier, HR-HPVs have evolved a strategy by which to overcome p53-mediated cell cycle control. The E6 oncoprotein binds to a short LxxLL consensus sequence within the cellular ubiquitin ligase, E6AP, forming a heterodimer (Huibregtse *et al.* 1991; Martinez-Zapien *et al.* 2016). A trimeric complex is subsequently formed by the recruitment of p53, leading to ubiquitin-dependent p53 proteasomal degradation (Scheffner *et al.* 1993). Using a combination of RNA interference and pharmacological induction of p53 protein with Nutlin-3 in breast cancer cell lines, they elucidated a mechanism whereby p53 represses A3B expression via the action of its target gene, $p21^{WAF1/CIP1}$ (*CDKN1A*) in stabilizing the E2F4/DP1/p107/p130-containing DREAM (DP1, RB-like, E2F4, and MuvB) transcriptional repressor complex (Fischer *et al.* 2014) at cell cycle genes homology region (CHR) elements in the A3B promoter. They also demonstrated that both the E6 and E7 proteins from HPV16 can act independently to increase A3B expression in immortalized keratinocytes through this pathway; E6 via p53 degradation, with E7 likely acting through its effects on the p107 and p130 pRb family pocket proteins in the DREAM complex (Periyasamy *et al.* 2017), thus also offering a mechanistic basis for the E7-mediated A3B upregulation previously observed by Warren and colleagues (Warren *et al.* 2015a).

The A3B promoter also harbours target elements for the TEAD family of transcription factors (TEAD1-4 in mammals) (Mori *et al.* 2017). These evolutionarily conserved transcription factors, that recognise the consensus DNA sequence (AGGAATG) mediate expression of multiple genes involved in cell proliferation, epithelial–mesenchymal transition and apoptosis evasion, acting in complexes with TAZ (transcriptional co-activator with PDZ binding motif) or YAP (Yes-associated protein), both of which are phosphorylated and inhibited by the Hippo tumour suppressor pathway (Jacquemin *et al.* 1996; Zhao *et al.* 2008; Zhang *et al.* 2009; Zhu *et al.* 2015). E6 induces TEAD1 and TEAD4 expression in keratinocytes and increases YAP protein levels by preventing it's degradation, although the TEAD-dependent induction of A3B

appears to be YAP/TAZ-independent and may instead involve alternative coactivators (He *et al.* 2015; Mori *et al.* 2017).

E6 mediated p53 degradation therefore not only de-represses A3B transcription via the DREAM complex but also results in increased levels of TEAD expression, further activating the A3B promoter. Finally, it has been reported that replication stress induced by oncogenic pathway activation or by chemotherapy agents such as hydroxyurea or gemcitabine also causes ATR/CHK1-dependent upregulation of A3B, at least in breast cancer cell lines (Kanu *et al.* 2016). High E6 / E7 levels might similarly drive A3B upregulation via this as-yet undefined replication stress mechanism, thus together with the ZNF384-mediated effects and the additional activity of E7 in potentiating A3B expression, it appears that HPV could upregulate A3B via multiple mechanisms. Importantly, some of these mechanisms may act during the productive life cycle, while others may be restricted to precancerous/cancerous cells in which HPV has integrated into the host genome, the life cycle has been aborted and only high-level E6/E7 expression remains. It is also worth noting that in cells with wild-type *TP53*, A3B over-expression induces ATR/CHK1-dependent cell cycle arrest and apoptosis (Nikkilä *et al.* 2017). By removing p53 then, HPV not only activates A3B transcription but possibly also allows the A3B protein to accumulate to levels that would not otherwise be tolerated in normal cells.

Although the regulation of A3B by HPV has been the focus of much attention, it is important to consider the roles that other A3 genes may play, both in the response to HPV infection and potentially, in HPV-associated cancer. In their key paper reporting the first evidence for APOBEC editing of HPV in human cells, Vartanian and colleagues noted that HPV1a DNA co-transfected with A3A, A3C and A3H but not A3B displayed evidence of cytosine deamination (Vartanian *et al.* 2008), and while low risk HPV genomes isolated from warts display evidence of A3 editing, several tested low risk E6 variants did not upregulate A3B in cultured keratinocytes (Vieira *et al.* 2014).

Taken together with the findings of Warren and colleagues, that A3A but not A3B inhibits HPV infectivity, we should at least consider the possibility that the A3 response to HPV infection is entirely separate from any role in host mutagenesis during cancer development, with the former mediated by A3A and/or A3C, A3H and the latter mediated by A3B. An alternative hypothesis is that although A3B is induced by HPV, it is not responsible for the mutations seen in either viral or host genomes. Consistent with this possibility is work from

the Gordenin lab showing that, at least when expressed in yeast A3A and A3B generate subtly different mutation signatures, in which A3A preferentially targets YTCA sites (i.e. a pyrimidine at the -2 position) while A3B targets RTCA (i.e. a purine at the -2 position). Upon analysis of tumour exome data, they found much greater enrichment of the YTCA (A3A) signature across multiple tumour types including cervical cancer (Chan *et al.* 2015). The apparent preference of A3A for pyrimidine at -2 is also supported by in vitro studies using purified enzyme (Shi *et al.* 2017; Silvas *et al.* 2018). These observations suggest A3A, rather than A3B, may be the major source of somatic mutations to the host genome in HPV-associated cancer, although further functional investigation (e.g. analysis of A3 signature mutation accumulation in A3A or A3B knockout cells expressing HPV oncogenes) will be required to help solve this question.

Additional cellular signalling pathways linked to A3 regulation

The appearance of the A3 mutational signature in genomes of cancers with (presumably) no viral aetiology clearly implicates alternative mechanisms for A3 induction. In addition to the p53-dependent repression and ATR/CHK1-dependent induction of A3B discussed above, several additional cellular pathways have been shown to induce A3 expression and it is worth considering how they may contribute to A3 activity against viral or host genomes in HPV infected cells.

Protein kinase C (PKC) signalling. The twelve PKC isoforms regulate a plethora of biological processes and are characterised as conventional/classical (cPKC), novel (nPKC), or atypical (aPKC). Receptor-mediated activation of phospholipase-C gamma (PLC) causes hydrolysis of the plasma membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into diacylglycerol (DAG) and inositol trisphosphate (IP3), with the latter stimulating release of intracellular Ca^{2+}. Both DAG and Ca^{2+} are required for cPKC activation, while nPKC activation is DAG-dependent but Ca^{2+}-independent (reviewed in Mellor and Parker, 1998; Newton, 2003). By mimicking DAG, the phorbol ester, phorbol 12-myristate 13-acetate (PMA, a tumour promoter in animal models) potently activates both the cPKCs and nPKCs. Depending on the cell type examined, both A3A and A3B induction has been reported upon activation of PKC signalling: A3A was originally identified as Phorbolin-1, a protein enriched in psoriatic keratinocytes that could be induced by treatment of normal keratinocytes with PMA (Rasmussen & Celis 1993;

Madsen *et al.* 1999), while A3B but not A3A, is induced following PMA treatment of the mammary epithelial cell line, MCF10A (Leonard *et al.* 2015). Conversely, while a recent study conducted in normal oral keratinocytes (Siriwardena *et al.* 2018) and our own observations using NIKS in which we have epitope-tagged the endogenous A3A and A3B genes (Smith and Fenton, unpublished) confirm a strong, protein kinase C (PKC)-dependent increase in A3A protein expression upon PMA treatment, A3B mRNA is induced to a far lesser extent in keratinocytes, with minimal or no detectable increase in A3B protein.

PKC isoforms perform important functions in keratinocyte proliferation and differentiation (Dlugosz & Yuspa 1993; Denning *et al.* 1995; Papp *et al.* 2003; Yang *et al.* 2003; Seo *et al.* 2004) and in the context of HPV infection, PKC-α and PKC-δ are required for high risk HPV-31 genome amplification during the intermediate phase of viral replication, while expression of E5 from HPV-16 in mouse fibroblasts causes PKC activation through activation of PLC γ (Crusius *et al.* 1999; Bodily *et al.* 2006). Interestingly, both A3A and A3B were recently shown to be upregulated during Ca^{2+}-stimulated differentiation of W12 cells (Wakae *et al.* 2018) and although the intracellular pathway mediating Ca^{2+}-induced A3A/B upregulation was not investigated in this study, it is well-established that increases in extracellular Ca^{2+} trigger activation of PKCs via PLC in keratinocytes (Jaken & Yuspa 1988). Activation of PKC signalling during differentiation of HPV-infected keratinocytes is therefore a likely means by which at least A3A and possibly also A3B could become upregulated during productive HPV infections, potentially triggering viral genome editing alongside amplification.

Viral nucleic acid sensing / interferon signalling. Antiviral responses can be triggered through the sensing of foreign DNA in endosomes by a subset of Toll-like receptors (TLRs), or in the cytoplasm by the cyclic GMP-AMP synthase (cGAS) / stimulator of interferon gene (STING) pathway (Lebre *et al.* 2007; Suspène *et al.* 2017). Both pathways result in the induction of type-1 interferons (IFNs), which in turn induce a host of interferon-stimulated genes (ISGs, including several A3s) with a broad range of antiviral activities, and both are inhibited by HPV, suggesting a role in sensing the virus (Hasan *et al.* 2007; Albertini *et al.* 2018). Human keratinocytes express several TLRs, among which TLR9 is activated by DNA containing unmethylated CpG motifs including a region from the HPV16 E6 gene (Hasan *et al.* 2007, 2013; Lebre *et al.* 2007). In addition to type 1 interferons it induces tumour necrosis factor (TNFα), which has recently been shown to upregulate A3A in keratinocytes (Amcheslavsky *et*

al. 2004; Siriwardena *et al.* 2018). The suppression of TLR9 by E7 provides further evidence of its importance in the innate immune responses to HPV (Hasan *et al.* 2007, 2013).

Both cGAS and the retinoic acid-inducible gene I (RIG-I, a sensor of viral RNA) have been implicated in keratinocyte responses to HPV infection and RIG-I is required for induction of A3A expression by cytoplasmic DNA in the monocytic leukaemia cell line, THP-1 (Suspène *et al.* 2017; Albertini *et al.* 2018; Chiang *et al.* 2018). Until recently it was thought that sensing of viral DNA was limited to the cytoplasm or endosome, however, sensors of nuclear viral DNA (IFI16, recently reported to restrict HPV18 replication (Lo Cigno *et al.* 2015) and IFIX) have been described that also act together with cGAS to induce IFN responses (reviewed in Diner, Lum and Cristea, 2015), thus providing another mechanism by which A3 activity could be induced in HPV-infected cells. Finally, HPV16 genome integration triggers a type I IFN response in keratinocytes, leading to episome clearance, loss of E2 expression and therefore upregulation of E6/E7 expression from the integrated virus (Pett *et al.* 2006). Whether viral integration is accompanied by IFN induction *in vivo* remains unknown but if so it could generate a burst of A3 expression in neoplastic cells consistent with the proposed pulsatile nature of the APOBEC mutational process (Helleday *et al.* 2014).

Downstream of viral nucleic acid sensing and PKC pathways lie NFκB transcriptional complexes known to participate in regulating A3B expression (Leonard *et al.* 2015; Maruyama *et al.* 2016). NFκB complexes are also directly activated by HR-E6; they become progressively activated during cervical cancer development (Nees *et al.* 2001; James *et al.* 2006; Da Costa *et al.* 2016; Tilborghs *et al.* 2017) and therefore likely contribute to the high A3B expression levels seen in these and other HPV-associated tumours. Finally, while little is yet known about how the A3 proteins are regulated, A3A and A3C have both been reported to bind the pseudokinase, TRIB3. TRIB3 is localised to the nucleus and appears to target nuclear A3A for degradation, thus inhibiting deamination of genomic DNA upon transfection of A3A into Hela cells (Aynaud *et al.* 2012). Knockdown of TRIB3 expression also increased A3A levels in NIKS but without an apparent stabilization of the protein (Westrich *et al.* 2018), while in a third study, Land and colleagues saw no effect of TRIB3 on A3A-GFP levels in HEK293T cells but did not report whether the A3A-TRIB3 interaction (initially observed in a yeast-2-hybrid screen) still occurred (Land *et al.* 2013). The fusion of GFP to A3A could possibly

explain the absence of TRIB3 regulation in the latter study but further work is required to determine the significance of the A3A-TRIB3 interaction.

Current questions

We have seen that multiple A3s can be induced in HPV-infected cells, and that circumstantial evidence supports a role for either or both A3A and A3B in generating mutations in host and viral genomes. Other A3s (A3C, A3G and A3H) all remain potential candidates, at least for viral genome editing. One outstanding question is whether viral and host genome editing are linked events, mediated by the same A3 at the same time. In this model, HPV induces A3 activity as discussed, possibly to generate variation in viral progeny, suppress retroelement replication-induced interferon responses or to mediate transcriptional functions allowing suppression of TLR9 expression or induction of DNA replication. Due to other activities of the virus however, such as the induction of replication stress, A3 activity against the host genome can also occur and in rare circumstances, this results in mutations in cancer-causing genes such as *PIK3CA*. Cells in which these mutations occur will gain a selective survival advantage but may remain held in-check by the host immune system for many years and / or lack additional genetic or epigenetic changes required to form an invasive carcinoma. In this scenario, A3 activity may contribute to tumour development even from the earliest stages of an HPV infection. This scenario is represented in Figure 4 as 'Early, transient' or Early, sustained' temporal models of A3 activity, depending on whether tumour subclones in which A3 mutagenesis has occurred early sustain high A3 activity, or whether this is subsequently selected against due to increased chance of deleterious mutations and/or generation of neoantigens and therefore immune-mediated elimination ('cancer immuoediting' (Schreiber *et al.* 2011)). It is noteworthy that HPV genomes are physically tethered to fragile sites in the host genome via the chromatin modifier, BRD4 (Jang *et al.* 2014), thus their replication (and likely A3 editing) occurs in very close proximity to host DNA, potentially increasing the danger of off-target A3 activity, particularly during the stable maintenance phase of viral replication which unlike amplification occurs concurrently with cellular genome replication in S-phase (Sakakibara *et al.* 2013; Reinson *et al.* 2015). Alternatively, the initial A3 response to viral infection may result in editing of HPV but not host DNA, with aberrant activity against the host genome coming much later, for example induced by IFN signalling associated with episome clearance or subsequent upregulation of E6/E7 from integrants due to loss of E2 expression. Either way, the genomic instability caused by high level E7 expression, together

555 with removal of p53 by E6 and chronic activation of NF-κB could all fuel A3 mutagenesis
556 throughout tumour development, not necessarily mediated by the same A3(s) responsible for
557 the viral editing seen in benign lesions. This scenario is represented in Figure 4 as 'Late,
558 transient' or 'Late sustained' A3 activity, again depending on whether A3 mutagenesis is
559 ongoing at the time of diagnosis and subsequently during treatment (see below). Given the
560 mutational and gene expression data currently available it is difficult to say which of these
561 models is the more likely, not least because almost all these data come from resected primary
562 tumour samples (Figure 4). At least in the case of cervical cancer it is possible to study
563 precancerous lesions, affording a rare opportunity to address some of these questions; a
564 targeted NGS study in which a panel of 48 cancer-associated genes including *PIK3CA* were
565 sequenced in 35 cervical cancers and 23 CIN2/3 lesions found only the *PIK3CA* exon 9 (A3-
566 mediated) mutations were detectable in the CIN2/3 lesions, with the rest exclusive to invasive
567 carcinoma. *PIK3CA* exon 9 was then Sanger-sequenced in a further 35 cervical carcinomas,
568 209 CIN3, 144 CIN2, 154 CIN1 and 105 normal samples, with mutations detected in 37% of
569 carcinomas but only 2.4% of CIN3 lesions and none in earlier lesions or normal cervix, leading
570 the authors to conclude this is a late event in cervical carcinogenesis (Verlaat *et al.* 2015). It is
571 important to note however, that Sanger sequencing would not have permitted the detection of
572 *PIK3CA* mutations in minor sub-clones that could be present from a much earlier stage in
573 carcinogenesis, thus ultra-deep sequencing of CIN lesions will be required to fully address this
574 question.
575
576 Analysis of allele frequency (a measure of the clonality, or proportion of tumour cells in which
577 a mutation is found and therefore a proxy for the time at which it occurred) for cancer driver
578 mutations seen in TCGA WES data suggests that A3 signature mutations become increasingly
579 enriched in later stages of tumour development in several tumour types. A3 mutations in lung
580 adenocarcinoma are largely subclonal (i.e. those occurring later), often 'taking over' from the
581 tobacco-associated signature, which generates clonal driver mutations (i.e. initiating or early
582 events) in smokers, consistent with its role in lung carcinogenesis (de Bruin *et al.* 2014;
583 McGranahan *et al.* 2015). In bladder cancer, A3 signature mutations appear in pre-invasive
584 tumours but continue to accumulate through progression, becoming increasingly enriched in
585 muscle-invasive disease, as confirmed by sequencing of matched metachronous samples
586 (Nordentoft *et al.* 2014; Lamy *et al.* 2016). Interestingly in bladder cancer it appears that one
587 of the two A3 mutation signatures defined by Alexandrov and colleagues (signature 13) is
588 enriched early, while the other (signature 2) becomes enriched in subclones (Alexandrov *et al.*,

2013a; McGranahan *et al.*, 2015). The reason for this is unclear but may reflect differences in DNA replication across deaminated sites in early versus late tumours. In breast cancer, there is evidence that A3 signature mutations begin to accumulate prior to copy number changes and that A3-generated mutation clusters ('kataegis') appear at several distinct stages during the development of a single tumour, again implying pulses of A3 activity from an early point, represented in Figure 4 as a 'Pulsatile' model for A3 mutagenesis (Nik-Zainal *et al.*, 2012a; Nik-Zainal, *et al.*, 2012b; Helleday, Eshtad and Nik-Zainal, 2014).

Maybe more important than the question of when A3 activity against the host genome first appears, is whether it is ongoing at the time of diagnosis (as represented by the 'Early, sustained', 'Late, sustained' and 'Pulsatile' models for A3 mutagenesis set out in Figure 4). Experiments in cultured cells suggest that acquired resistance to cancer therapy can occur both by selection of rare, pre-existing drug-resistant subclones and *de novo* mutations in 'drug-tolerant' cells (Hata *et al.* 2016; Ramirez *et al.* 2016), and evidence that A3 activity continues to generate mutations during treatment is accumulating, both from sequencing of metastatic bladder cancer, post-gemcitabine/cisplatin-based chemotherapy (Faltas *et al.* 2016) and from experimental models, in which chemotherapy drugs including gemcitabine have been shown to induce A3B expression and deaminase activity via ATR/CHK1 signalling (Kanu *et al.* 2016). These observations suggest A3 activity could contribute to the evolution of therapeutic resistance, a possibility that is supported by a recent study in which suppression of A3B expression by inducible RNA interference delayed the acquisition of tamoxifen resistance in a xenografted breast cancer cell line (Law *et al.* 2016). Increased A3B expression is also associated with shorter overall survival and progression-free survival in patients receiving Tamoxifen treatment in ER+ breast cancer (Sieuwerts *et al.* 2014; Law *et al.* 2016). It appears then, that there could be therapeutic benefit to be gained through inhibiting A3B and/or other A3 enzymes as an adjuvant to chemotherapy; a notion underlying A3 drug discovery efforts currently underway in academia and industry (Olson *et al.* 2018; Venkatesan *et al.* 2018). In addition to suppressing *de novo* mutagenesis and therefore the emergence of drug-resistant subclones, an inhibitor of A3B could also have anti-cancer effects by interfering with its activity as an ER transcriptional co-activator in breast but possibly also in other eostrogen-responsive tissues including the cervix as discussed earlier and ovarian cancer, another malignancy in which A3B activity has been implicated (Leonard *et al.* 2013).

One important consideration for developing A3 inhibitors as cancer therapies is whether a selective inhibitor would be preferable to a pan-A3 inhibitor and if so, which would be the best A3 to target. A rationale for selectively targeting A3B comes from the fact that it is a non-essential gene in humans, as evidenced by the existence of a deletion polymorphism (A3A_B) in which the A3A 3' untranslated region (UTR) and entire A3B open reading frame (ORF) are absent and the A3A ORF is fused to the A3B 3' UTR (Figure 2). This polymorphism displays a remarkable stratification across the global population, with a prevalence of 1% in Africa rising to approximately 40% in South East Asia and South America and approaching fixation in Oceania (Kidd *et al.* 2007). Somewhat surprisingly given the demonstrated mutagenic and pro-growth functions of A3B in breast cancer cell lines, this deletion allele is associated with an approximately 2-fold increased breast and ovarian cancer risk in Asian populations and in certain European cohorts (Long *et al.* 2013; Xuan *et al.* 2013; Qi *et al.* 2014; Middlebrooks *et al.* 2016; Wen *et al.* 2016). A recent Scandinavian study meanwhile, found an increased lung cancer risk in A3A_B carriers aged under 50 and a similar age-related trend for prostate cancer risk but no association with breast cancer risk, a result consistent with a further study conducted in Sweden (Göhler *et al.* 2016; Gansmo *et al.* 2017). The reason for the increased cancer risk associated with A3A_B remains unclear but it was shown that breast cancers from women carrying at least one copy of the deletion allele harbour an increased burden of A3-related mutations, suggesting another A3 enzyme is hyper-activated in these tumours (Nik-Zainal *et al.* 2014). A hybrid A3A transcript encoded by a recombinant cDNA based on the A3A_B allele accumulates to levels approximately 2-fold higher than those of A3A bearing its own 3'UTR in transient transfection experiments and in a Taiwanese oral squamous cell carcinoma (OSCC) cohort, A3A was upregulated at both mRNA and protein levels and the A3 mutation signature was enriched in the 50% of patients carrying the A3A_B allele (Caval *et al.* 2014; Chen *et al.* 2017). Another study however proposed that mutations in A3A_B tumours are generated by a specific variant of the polymorphic A3H gene. This variant (A3H haplotype I) encodes a less stable but nuclear-localised protein that does not display linkage disequilibrium with A3A_B but those A3A_B homozygous breast tumours with the highest A3 signature mutation loads in TCGA cohort were found to be hetero- or homozygous for A3H-I (Starrett *et al.* 2016). An A3B-selective inhibitor would therefore not be expected to display on-target toxicity, although it would clearly be ineffective in A3A_B patients. In the Taiwanese OSCC study, A3A expression was associated with longer disease-specific, disease-free and overall survival specifically in those patients hetero- or homozygous for A3A_B, again supporting a key role for A3A in these tumours (Chen *et al.* 2017). The authors of this study found that A3A

expression was reduced in tumours of higher stage but A3A expression was nevertheless significantly associated with both overall and disease-specific survival in a multivariate analysis including clinicopathological variables such as age, tumour stage, grade, evidence perineural or bone invasion, in A3A_B carriers. The magnitude of this effect was marked, with a disease-specific survival hazard ratio of 0.444 for 'A3A-high' tumours versus 'A3A-low' tumours. It is possible that the improved survival in this group could be linked to increased neoantigen loads and therefore an enhanced adaptive immune response, as recently posited for lung cancer, in which tumours with higher A3B levels displayed greater immune infiltration and more durable responses to immune checkpoint blockade (Wang *et al.* 2018) and for bladder cancer, in which a higher A3 signature mutation load was associated with improved prognosis (Middlebrooks *et al.* 2016). It is also possible that the A3A expression detected in the Taiwanese OSCC cohort emanated from infiltrating leukocytes rather than the tumour cells, thus serving as a marker of immune infiltration. In this regard, it is interesting that the A3A_B allele has previously been linked to increased immune infiltration in breast cancer (Cescon *et al.* 2015; Wen *et al.* 2016). More studies on the expression and activity of the A3 enzymes in A3A_B cells and tumours will be required to resolve these questions and more epidemiological studies are needed to investigate potential associations between A3A_B and risk of other cancers, including HPV-associated cancer. Given that A3A and A3B are induced by HR-HPV, that HPV-associated cancers display such strong enrichment for the A3 mutational signature and that A3 activity appears to generate within-host sequence variation in the viral genome, we might expect A3A_B to confer an altered risk, either of persistent HPV infection and / or carcinogenesis, and possibly even prognosis.

Conclusions

One or more of the A3 genes play important roles in the development of HPV-associated cancers, by generating somatic mutations in the host genome but potentially also via their activity against the virus. Our current understanding of A3-mediated mutagenesis in tumour cells stems in large part from analysis of cancer sequencing data, supported by studies in which A3 enzymes have been expressed either in human or yeast cells. The prospects of utilising A3s as predictive biomarkers for cancer immunotherapy or targets for cancer treatment are tantalising but much remains to be learned regarding which A3s are the most important players in different cancers and how they become deregulated. To address such questions, we will need to develop and utilise models in which we can study individual A3 genes in relevant models of

HPV infection and carcinogenesis. The relative ease of conducting loss-of-function studies enabled by CRISPR-Cas9 technology (Shalem *et al.* 2015) should facilitate progress in this regard, as will the chemical probes that we hope will soon emerge from A3 inhibitor programmes (Olson *et al.* 2018). Studying A3 function in animal models remains a challenge but approaches such as the expression of A3 transgenes in a mA3-null background provide useful proof-of-concept and good mouse models of E6/E7-driven carcinogenesis are available (Riley *et al.* 2003; Strati *et al.* 2006; Stavrou *et al.* 2014). In conclusion, the exploration of A3 involvement in cancer is still a new field, much remains unknown and we anticipate many exciting developments in the coming years.

Declaration of Interests

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1: HPV16 genome organisation and replication cycle. (A) The HPV16 genome is shown, with the E6 and E7 oncogenes represented in red, the remaining early genes in orange and the genes encoding the major (L1) and minor (L2) capsid proteins shown in green. The origin of replication in the long control region (yellow star) appears to be most heavily edited by one or more human A3 enzymes. **(B)** The productive HPV replication cycle in stratified epithelia: (1) Virus entry (purple dots) to the basal layer at the site of an abrasion (for example in the stratified epithelium of the ectocervix) is shown; (2) infection of basal cells is followed

by expression of E6 and E7 (red), leading to host cell cycle entry and initial replication of the HPV genome during S-phase; (3) cells in the mid-layer enter differentiation and are held in an extended G2 phase during which HPV genomes are amplified by the host cell homology-directed repair machinery (orange colour represents expression of HPV early genes E1, E2, E4, E5); (4) virus assembly occurs in the upper layers, in which early gene expression is replaced by L1 and L2 (green) during terminal differentiation and enucleation of host keratinocytes; (5) viral particles (purple dots) are released from the epithelium (adapted from Doorbar *et al.* 2012; Lechner & Fenton 2016).

Figure 2: Schematic representation of the *APOBEC3* locus in humans and mice. Approximate relative lengths of the open-reading frames (ORFs) and 3' untranslated regions (UTRs) of each gene are shown and homologous domains are represented in common colours. The A3A_B deletion polymorphism is also represented, showing the fusion gene in which the A3A ORF is fused to the A3B 3'UTR (relative 3' UTR sizes from UCSC Genome Browser (https://genome.ucsc.edu/).

Figure 3: Regulation of APOBEC3A and APOBEC3B gene expression by several cellular pathways impacted by HPV E6 or E7. Pathways implicated in regulation of A3A and A3B transcription are shown, grey arrows indicate proposed regulation via unknown intermediates, dashed lines represent transcriptional regulation of the gene encoding the target protein, see main text for details.

Figure 4: A clonal selection model for HPV-associated tumour development and progression, with alternative models for temporal involvement of A3 activity. Top panel: following persistent HPV infection, somatic alterations begin to accumulate in the host cell genome, resulting in clonal expansion and the appearance of multiple subclones (increased genetic variation, y-axis). Typically, cells in which viral integration occurs at particular site(s) in the host cell genome will outgrow surrounding neoplastic clones, resulting in an invasive carcinoma that is diagnosed and removed. The vast majority of information regarding somatic mutations, mutational signatures and A3 gene expression currently comes from samples taken at this point, thus it is not clear when A3 mutagenesis occurs during tumour development, whether it is ongoing at the time of diagnosis and treatment and how it contributes to adaptation to continual selection pressures or selective sweeps such as those shown in black dashed lines (immunoediting or radiotherapy / chemotherapy respectively). Bottom panel: alternative

models for the temporal pattern of A3 activity (y-axis) against the host genome during HPV-associated tumour development and progression (see main text for details). Note that the y-axis represents 'mutagenic A3 activity' and not the expression of any one A3 enzyme. It is possible that different A3s could be active at different stages of tumour development.

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1267

FIGURE 1

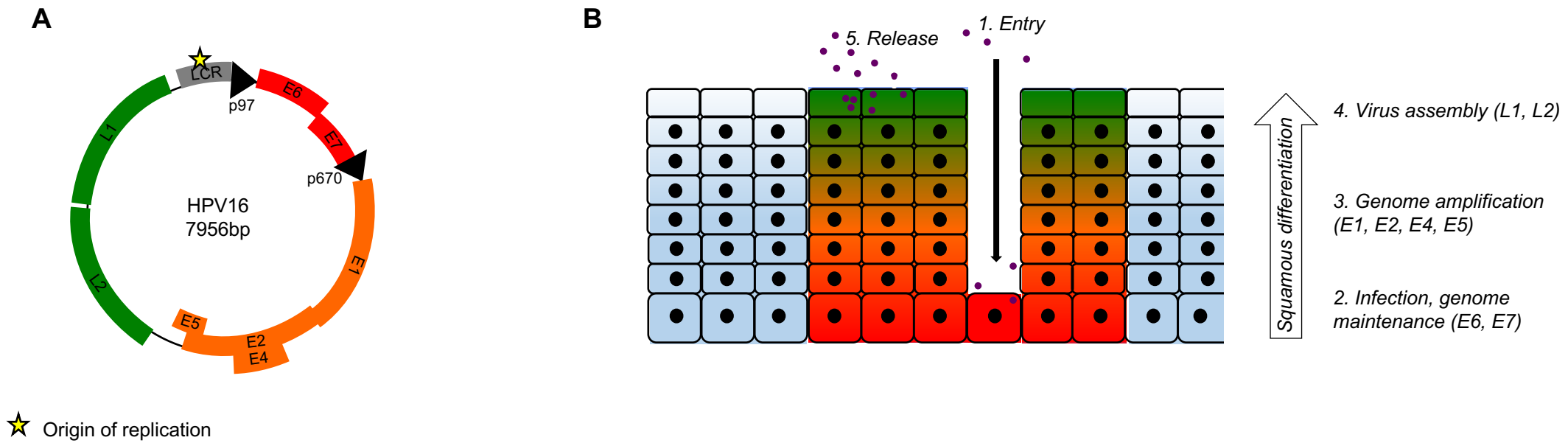

A
LCR
p97
E6
E7
p670
HPV16
7956bp
L1
L2
E5
E2
E4
E1
Origin of replication
B
5. Release
1. Entry
Squamous differentiation
4. Virus assembly (L1, L2)
3. Genome amplification
(E1, E2, E4, E5)
2. Infection, genome
maintenance (E6, E7)

FIGURE 2

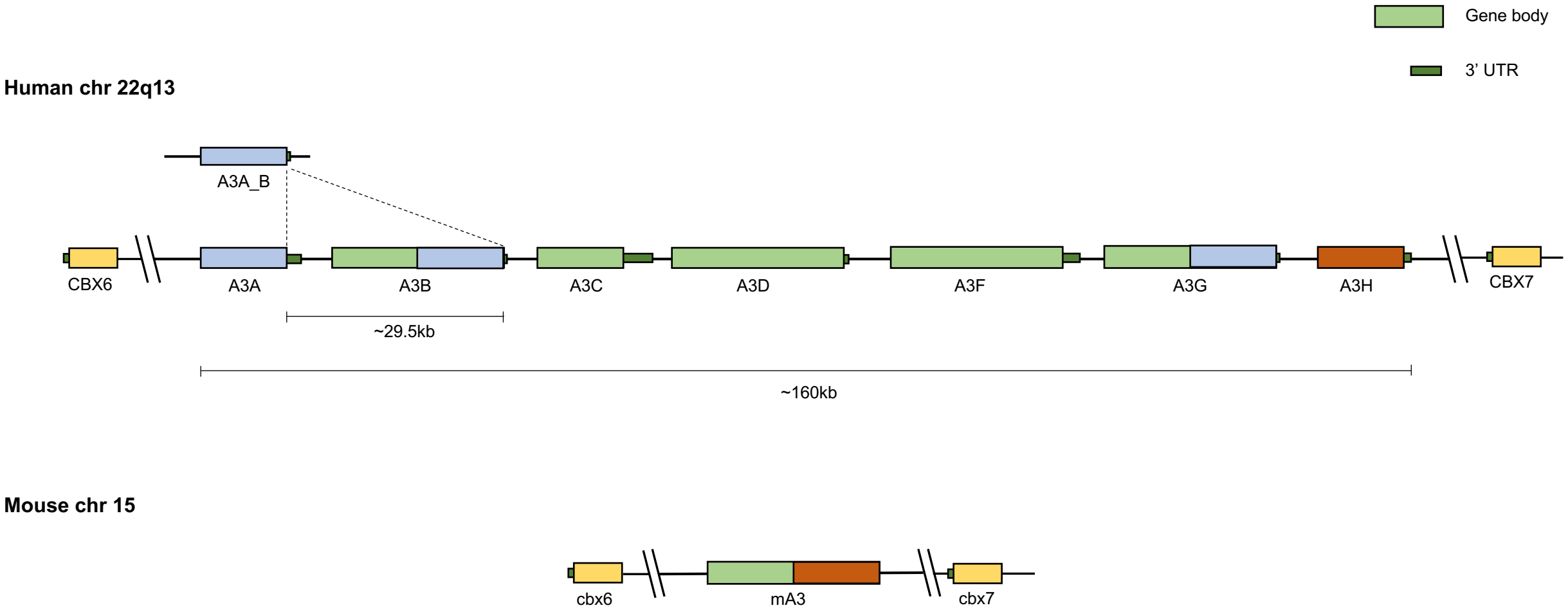

Gene body
3' UTR
Human chr 22q13
A3A_B
CBX6
A3A
A3B
A3C
A3D
A3F
A3G
A3H
CBX7
~29.5kb
~160kb
Mouse chr 15
cbx6
mA3
cbx7

FIGURE 3

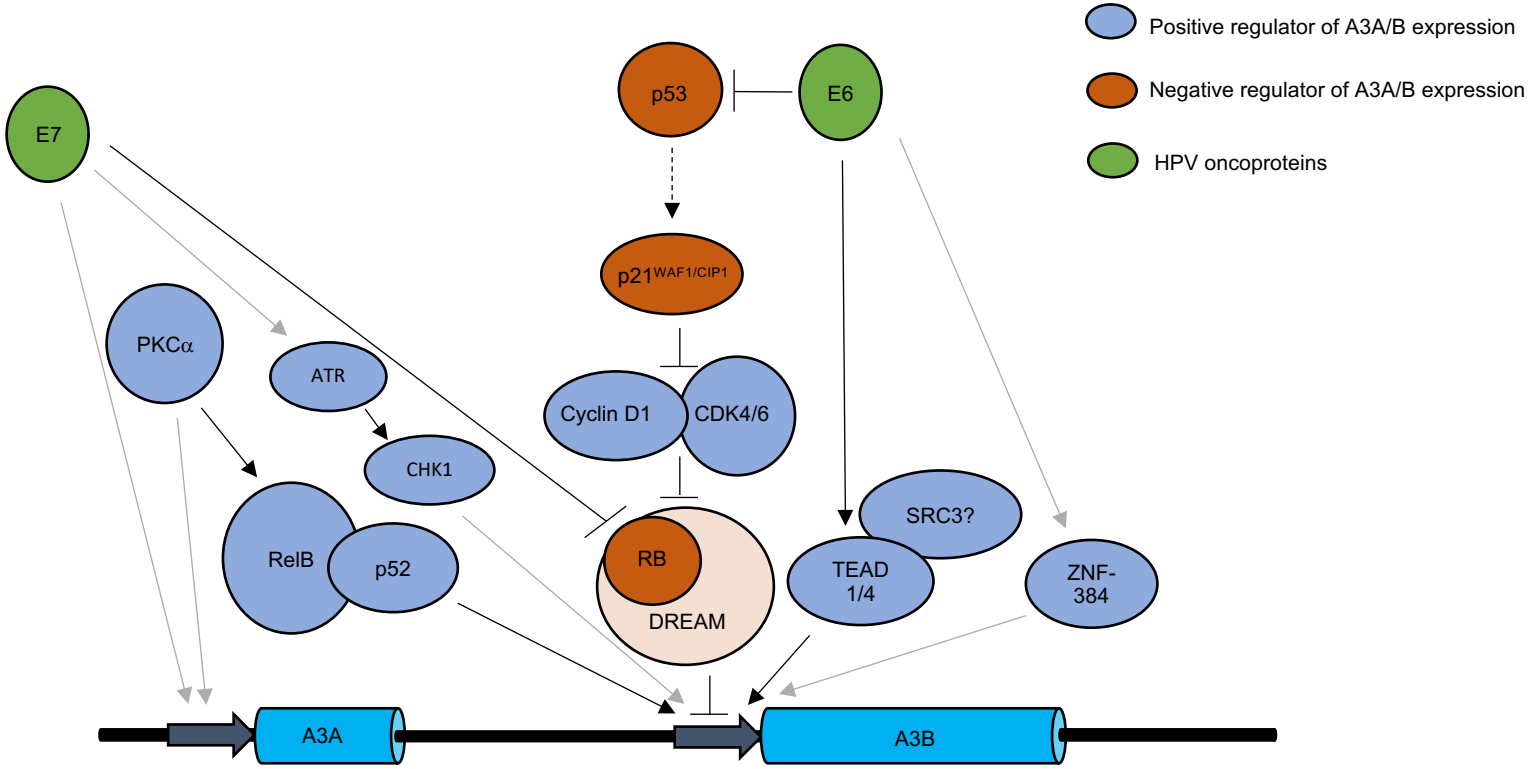
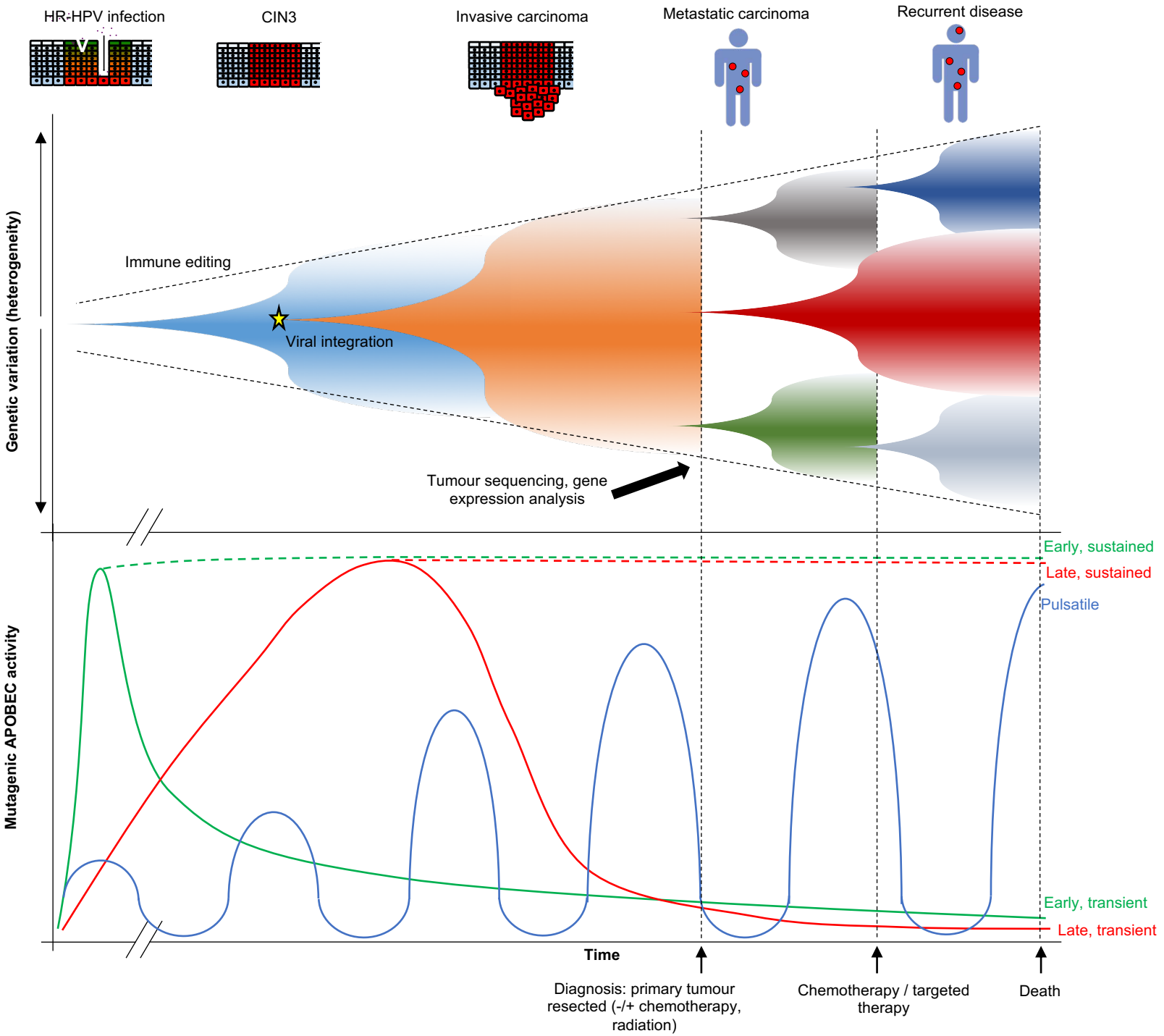

Positive regulator of A3A/B expression
Negative regulator of A3A/B expression
HPV oncoproteins
p53
E6
E7
p21WAF1/CIP1
PKCα
ATR
Cyclin D1
CDK4/6
CHK1
SRC3?
RelB
p52
RB
TEAD 1/4
ZNF-384
DREAM
A3A
A3B

FIGURE 4


HR-HPV infection
CIN3
Invasive carcinoma
Metastatic carcinoma
Recurrent disease
Genetic variation (heterogeneity)
Immune editing
Viral integration
Tumour sequencing, gene expression analysis
Mutagenic APOBEC activity
Early, sustained
Late, sustained
Pulsatile
Early, transient
Late, transient
Time
Diagnosis: primary tumour resected (-/+ chemotherapy, radiation)
Chemotherapy / targeted therapy
Death