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At a crossroads: human DNA tumor viruses and the host DNA damage response

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Abstract

Human DNA tumor viruses induce host cell proliferation in order to establish the necessary cellular milieu to replicate viral DNA. The consequence of such viral-programmed induction of proliferation coupled with the introduction of foreign replicating DNA structures makes these viruses particularly sensitive to the host DNA damage response machinery. In fact, sensors of DNA damage are often activated and modulated by DNA tumor viruses in both latent and lytic infection. This article focuses on the role of the DNA damage response during the life cycle of human DNA tumor viruses, with a particular emphasis on recent advances in our understanding of the role of the DNA damage response in EBV, Kaposi's sarcoma-associated herpesvirus and human papillomavirus infection.

Keywords

DNA tumor virus; EBV; Epstein–Barr virus; genomic instability; HBV; hepatitis B virus; HPV; human papillomavirus; human polyomavirus; Kaposi's sarcoma-associated herpesvirus; KSHV; oncogenic; stress; tumor suppression

The DNA damage response acts as an innate barrier to tumorigenesis

In recent years, the DNA damage response (DDR) has become recognized as an important innate tumor suppressor pathway [1,2]. Along with the ARF–p53 axis [3], the DDR is activated upon acute oncogenic stress in a variety of cell types and early neoplastic lesions [4–6]. A primary mechanism by which activated oncogenes induce the DDR is through increased replicative stress culminating in collapsed or arrested replication forks that are recognized as damaged DNA [1,2]. The ataxia-telangiectasia and RAD3-related kinase (ATR) signaling pathway primarily responds to ssDNA exposure following replication fork collapse, while processing of this damage to dsDNA breaks (DSBs) activates the ataxia-telangiectasia mutated kinase (ATM) pathway [7–9]. ssDNA coated by replication protein A (RPA) is recognized by ATR and ATR-interacting protein (ATRIP). ATR is activated following binding to RPA-ssDNA in the presence of topoisomerase binding protein

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(TOPBP)1 that interacts with the RAD9–RAD1–HUS1 (9–1–1) complex bound at the junction of the 5' primer and the ssDNA [10,11]. Downstream signaling to Chk1, p53 and other targets also requires the adaptor protein Claspin [12]. DSBs are recognized by the Mre11–Rad50–Nbs1 (MRN) complex, which promotes ATM activation at the sites of DNA damage [13]. Adaptor proteins 53BP1 and MDC1 among others stabilize DDR foci and are critical for downstream signaling [14,15]. Activated ATM phosphorylates downstream targets including the histone H2A variant H2AX, Nbs1, Chk2, p53 and CDC25 family members [11]. Signaling downstream of these initial sensors of DNA damage leads to the activation of cell cycle checkpoints and the initiation of DNA repair (Figure 1). Robust DNA damage induced by activated oncogenes often tips the normal cellular balance of checkpoint activation, triggering oncogene-induced senescence or apoptosis [1,2]. An activated DDR thereby constitutes an early barrier to cancer development (reviewed in [16]). However, continued flux through this pathway provides selective pressure in components of the DDR. Mutations in the sensors, kinases and downstream effectors in these pathways give rise to genomic instability and increased susceptibility to cancer [17].

Viruses & the DDR

The DDR is also emerging as a critical intrinsic sensor of viral infection [18]. Viral nucleic acid replication is inherently sensitive to recognition by the host DNA damage machinery either through frank damage of viral DNA or through aberrant exposure of DNA structures recognized by the DDR. Recruiting DNA repair factors could potentially have deleterious consequences for viral genomes, such as homologous recombination or inappropriate repair of viral DNA [19,20]. As a canonical example, adenovirus infection can be limited by the DDR, preventing proper viral DNA processing and enforcing concatemerization [20,21]. However, the viral E4orf6/E1B-55K protein complex promotes degradation of the DNA damage-sensing MRN complex [20]. This initial paradigm has illustrated the significance of the DDR as an intrinsic antiviral defense strategy and highlighted mechanisms by which viruses overcome this response to enable genome replication. In recent years, adenovirus and other DNA viruses, including herpesviruses, have also been shown to mislocalize DDR proteins or modulate DDR signaling, thereby derailing antiviral activities [22,23]. Despite the potentially antiviral downstream outcomes of these interactions, viruses can also benefit from aspects of DDR function and signaling [24]. For example, the γ -herpesvirus MHV68 is unable to replicate efficiently in the absence of the DDR scaffold H2AX [25]. Thus, viral proteins must engage these pathways with great precision in order to ensure viral DNA replication and cell survival during both latent and lytic phases of infection.

Human DNA tumor viruses impinge on the DDR through three distinct modes of interaction. First, viral oncoprotein expression may induce sufficiently robust cell proliferation to stress host DNA replication leading to activation of the DDR. Second, viral proteins expressed during either the latent or lytic cycles of infection are capable of directly interacting with and modulating the function of DDR components. Third, the replication of viral DNA generates structures that can be recognized by the DDR machinery. This review will discuss recent advances in our understanding of the interaction between DNA tumor viruses and each of these aspects of DDR activation with a particular emphasis on EBV, Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) and human papillomavirus (HPV).

EBV & the DDR

EBV is a human γ -herpesvirus that primarily infects B lymphocytes and epithelial cells and establishes life-long latency in the memory B-cell compartment of approximately 90% of adults worldwide, in the absence of clinical symptoms [26]. EBV is the cause of infectious mononucleosis as well as endemic African Burkitt's lymphoma (BL), most HIV-associated

B-cell lymphomas, a subset of sporadic Hodgkin's lymphomas, and the epithelial-derived nasopharyngeal carcinoma [26]. In immunocompromised patients following transplant, EBV can also drive a lymphoproliferative disorder that can develop into frank lymphoma.

EBV infection of normal human B cells *in vitro* transforms them into indefinitely proliferating, karyotypically stable, nontumorigenic lymphoblastoid cell lines (LCLs), which express a subset of viral latent proteins and small non-coding RNAs, collectively called latency III [26]. The proteins expressed are the EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and LP) and the latent membrane proteins (LMP1, 2A and 2B). EBNA1 is a DNA-binding transcription factor that primarily functions to replicate and faithfully segregate the EBV episome [27]. EBNA2 and EBNA-LP function as a collaborative transcriptional unit upregulating core cellular growth control genes including *c-Myc* [28]. The EBNA3 proteins modulate EBNA2-dependent gene expression and interact with other targets. Typically the EBNA3 proteins suppress EBNA2-driven transcription [29,30], but co-activation has also been reported for certain target genes such as the viral *LMP1* [31]. LMP1 is a constitutively active mimic of the TNF receptor and B-cell co-stimulatory molecule CD40 that triggers NF- κ B, p38 and JNK signaling pathways [32]. LMP2 proteins act as suppressors of endogenous B-cell receptor signaling while constitutively activating downstream tyrosine kinases to ensure high-level PI3K signaling and other survival pathways [33]. The viral latency III gene expression program observed in LCLs is also found in EBV-associated AIDS lymphomas and post-transplant lymphoproliferative disorder [26]. By contrast, more restricted latency expression profiles are found in Hodgkin's lymphoma and nasopharyngeal carcinoma (latency II: *EBNA1*, *LMP1*, *LMP2A*, *LMP2B*, *EBERs* and *BARTs*), BL (typically latency I: *EBNA1*, *EBERs* and *BARTs*), and in the memory B-cell compartment (essentially no gene expression) [26,34].

EBV can be reactivated from latency upon activation of the B-cell receptor or signaling downstream of phorbol esters in B cells [26,35]. During the lytic cycle, viral DNA replication is coupled to the synthesis of structural proteins and virion particle production occurs. Lytic replication also occurs during epithelial cell infection *in vivo* and following heterologous expression of the Zta lytic transactivator in EBV DNA-transfected 293 cells [26,36]. The DDR has been implicated in the regulation of both latent and lytic EBV infection.

EBV latent gene expression in primary B cells activates a transient growth suppressive DDR

The efficiency of B-cell transformation by EBV is low; approximately 1% of infected cells are immortalized [37,38]. Therefore, restriction of latency-driven B-cell proliferation is likely. Nikitin *et al.* recently demonstrated that at least approximately 10% of this host restriction is due to a viral latent gene expression program-provoked DDR (Figure 2A) [39]. Infection of primary human B cells by EBV induced hallmarks of an ATM-dependent DDR including phosphorylation of H2AX (γ -H2AX), the formation of nuclear foci of the DDR scaffold 53BP1, Ser1981-autophosphorylated and activated ATM, and Thr68-phosphorylated and activated Chk2 (Figures 1 & 2A). Using CFSE staining and flow cytometry to precisely measure B-cell proliferation, it was evident that EBV induced a transient period of hyperproliferation (one division per ~8–12h) followed by deceleration of the division rate to that found in immortalized LCLs (one division per ~24h). Furthermore, hyperproliferating cells displayed heightened DDR markers relative to subsequent divisions and LCLs. Importantly, cells in these divisions were nearly 100% latently infected based on EBNA-LP staining, whereas less than 1% were undergoing lytic DNA replication [39]. Also, despite evidence for the association between latently replicating EBV episomes and components of the DDR [19], the activated DDR markers in hyperproliferating cells did not co-localize with EBV episomes, strongly implicating host DNA damage [39]. Indeed, a

recent study of early EBV-infected primary B cells observed DNA damage associated with exposed telomeres and aberrant karyotypes, characterized by increased deletions, fragments and dicentric chromosomes [40]. Such structural abnormalities were not present in LCLs established from early cultures as previously described [40,41]. Therefore, early hyperproliferation of EBV-infected B cells induced a growth-suppressive and genotoxic DDR, while long-term outgrowth of immortalized LCLs displayed genomic stability.

The EBV-infected hyperproliferating cells were characterized by increased EBNA-LP protein, modest EBNA3 family protein expression, and high EBNA2-driven output, including *c-Myc* and CD23 [39]. In early cell divisions a switch occurs in viral promoter usage from the host transcription factor-driven Wp to the EBNA2-driven Cp [42]. This transition enables EBNA3 family proteins to accumulate, leading to attenuation of EBNA2/LP-driven gene expression including *c-Myc* transcripts and genome-wide *c-Myc* targets [39,42]. In fact, EBNA3C is specifically required for attenuation of the EBV-driven DDR [39]. EBNA3C has been shown to repress cyclin-dependent kinase inhibitors p14ARF and p16INK4A [43,44], as well as associate with a number of downstream effectors of the DDR [45]. Most interestingly, a recent report indicates that EBNA3C can directly associate with Chk2 and impinge on its function [46]. However, the precise role of EBNA3C in suppressing the DDR in early B-cell infection remains to be characterized.

The functional significance of the transiently EBV-activated DDR was revealed using pharmacological inhibitors of the ATM and Chk2 kinases. When present during the early hyperproliferative period, the inhibitors increased the efficiency of EBV immortalization nearly tenfold. Thus, consistent with observations in studies of early neoplasia of nonviral etiology, the DDR serves as a barrier to the EBV-mediated growth transformation of B lymphocytes [39].

EBV latent oncoproteins target multiple aspects of DDR signaling

Unlike the karyotypic stability displayed in EBV-transformed LCLs [41], BL cells often display evidence of genomic instability [47]. Given the role of EBV in BL pathogenesis, Massucci and colleagues analyzed mitotic chromosomes in EBV-positive and EBV-negative BL to assess the role of EBV gene products in this phenotype [47,48]. Strikingly, three viral proteins, EBNA1, EBNA3C and LMP1, were each independently capable of altering the frequency of chromosomal aberrations in BL cells (Figure 2B). However, the mechanisms by which these proteins induced genomic instability were distinct. EBNA1 activated a DDR through upregulation of the transcription factor NOX2, which increases levels of reactive oxygen species (ROS) in the cells heightening DNA damage and causing aberrant chromosomal structures such as fragments and dicentric chromosomes [49]. This phenotype could be completely reversed in the presence of anti-oxidants such as ebselen or citric acid [49]. Since EBNA1 is expressed in virtually all EBV-positive tumors, the overexpression of NOX2 may be a tumor-promoting mechanism worth targeting pharmacologically in addition to anti-oxidant-based therapies. In contrast to EBNA1, EBNA3C promoted genomic instability through its ability to disrupt the mitotic checkpoint [50], presumably through interactions with BubR1 [48]. Finally, high-level LMP1 expression suppresses DNA repair [51], which may be a result of suppressing expression of the DNA damage-sensing kinase ATM [48].

The diverse mechanisms by which EBV latency proteins activate or perturb the DDR may ultimately play an important role in the pathogenesis of EBV-associated tumors. During primary B-cell infection, a robust EBNA2-driven period of hyperproliferation may be mutagenic providing a milieu of cells within which overexpression of other latency proteins such as EBNA3C or LMP1 could lead to disruption of the DDR pathway. Such a scenario would be likely to drive B-cell tumorigenesis. In contrast, the *in vitro* LCL transformation

model generates a constitutively activated, but karyotypically stable, cell line expressing all of the latency III proteins [26,41]. Understanding the mechanisms that perturb latent gene expression *in vivo* promoting tumorigenesis is thus of the highest importance.

EBV episome replication requires DDR proteins

The EBV DNA episome is faithfully replicated and segregated once per latently infected cell cycle by EBNA1 [27]. The *cis*-acting element in the viral genome that EBNA1 binds is called oriP. EBNA1 binding to oriP facilitates interactions of the episome with cellular proteins involved in DNA replication and repair. In particular, viral DNA is replicated through EBNA1 association with the origin recognition complex and minichromosome helicases [52,53]. Within the dyad symmetry element of the oriP, EBNA1 binds four sites and telomere repeat binding factors TRF1, TRF2 and Rap1 bind at interspersed nonamers that resemble telomere repeats [54,55]. While TRF2 and Rap1 binding are critical for EBNA1-mediated replication, TRF1 is inhibitory [55]. Further, the poly-ADP ribosylating enzymes tankyrase and PARP1 associate with TRFs and inhibit efficient EBNA1 loading of the origin recognition complex and replication [56,57]. In addition to TRF binding factors, EBNA1 also recruits the DNA damage sensing MRN complex to oriP during S-phase [19]. The processing of a Holliday junction structure at oriP during S-phase appears to require MRN, as cells lacking these proteins integrate EBV DNA at high frequency [19]. Therefore, during latent EBV infection EBNA1 subverts the functions of DDR proteins to enable efficient replication and processing of the viral genome in the absence of overt checkpoint signaling.

DDR activation during EBV lytic reactivation

Lytic DNA replication activates an ATM signaling pathway including γ H2AX, pATM, pChk2 and phospho-p53 [58,59]. It is likely that viral DNA replication provides structures that activate ATM and induce the DDR (Figure 2C). In addition to replication of viral DNA, a recent study found that the major tegument protein BPLF1 is sufficient to induce an ATM/Chk2/ γ H2AX DDR pathway [60]. Interestingly, BPLF1 induces the DDR through deneddylation and stabilization of Cdt1, a key S-phase licensing factor (Figure 2C). BPLF1 overexpression triggers host hyper-DNA replication that also occurs during EBV lytic reactivation [60]. Thus, both viral DNA structures and BPLF1-mediated Cdt1 stabilization may contribute to DDR activation during the lytic phase of EBV infection [60].

As a means to suppress checkpoint activation downstream of the lytic-induced DDR, the key EBV lytic switch protein Zta physically interacts with p53 to suppress transactivation thereby preventing cell cycle arrest or apoptosis [61]. Interestingly, a recent study indicates that Zta also engages the DNA repair protein 53BP1 and this facilitates viral DNA replication [62]. Therefore, the EBV lytic cycle activates a host DDR that is counteracted by multiple mechanisms to enable efficient viral DNA replication and packaging (Figure 2C).

KSHV & the DDR

Kaposi's sarcoma-associated herpesvirus is a γ -herpesvirus that is able to establish viral latency and is causally implicated in several human tumors including KS [63] and AIDS-associated primary effusion lymphomas [64]. These tumors characterize two major reservoirs of KSHV infection, endothelial cells and B lymphocytes. While there is no transformation model for KSHV, latency can be established in human endothelial cell cultures and maintained in tumor cells derived from primary effusion lymphomas [65]. KSHV latently infected cells express v-cyclin, latency-associated nuclear antigen (LANA), viral FLICE inhibitory protein (vFLIP), kaposins, viral miRNAs, as well as some lytic replication products [66,67]. LANA maintains the viral episome [68] and interferes with

tumor-suppressive host pathways, including p53 [69–71] and E2F-Rb [72], as well as promoting activation of telomerase expression [73,74]. v-cyclin is a potent activator of cyclin-dependent kinase 6 and an inducer of genomic instability [75,76]. vFLIP activates NF- κ B, that provides survival signals through expression of antiapoptotic molecules and cytokine production [77,78].

KSHV endothelial cell infection activates the host DDR through its v-cyclin homolog

Ojala and colleagues observed that KSHV infection of human telomerase-immortalized primary dermal microvascular endothelial cells (hT-HDMECs) induced a growth-suppressive DDR characterized by centrosome abnormalities [79]. *In vivo* evidence from KS lesions indicated that early-stage (patch) KS lesions displayed high-level expression of DDR markers, while late-stage (nodular) lesions displayed attenuated DDR levels (Figure 3A). These findings are consistent with other tumors in which mutation or suppression of the DDR pathway provides a selective growth advantage [17,79].

KSHV latent proteins activate the host DDR, promote mutagenesis & modulate p53

To define the mechanism of KSHV-mediated DDR activation, v-cyclin expression in HDMEC cultures was observed to induce a robust host DDR including phosphorylation of ATM, Chk2, p53 and H2AX [79]. Interestingly, inhibition of ATM or Chk2 in v-cyclin-expressing cells did not increase proliferation, but rather led to mitotic catastrophe. This is thought to be due to v-cyclin-induced centrosome amplification, which, in the absence of DDR-mediated S-phase arrest, leads to aberrant mitosis [79–81]. Furthermore, v-cyclin has been shown to promote mutagenesis through v-cyclin–CDK6-mediated nucleophosmin phosphorylation, which may increase selection for mutations in growth-suppressive genes [79,81,82]. The effects of DDR activation on HDMEC proliferation could be rescued by inhibiting the ATM signaling pathway or through ablation of the Rb or p53 axes [79]. These results, together with the KSHV infection studies and analysis of KS lesions, strongly implicate the DDR as a true innate barrier to KSHV-infected cell proliferation and possibly KS pathogenesis.

The attenuation of the host DDR during KSHV latent infection suggests that viral proteins may function to suppress the inevitable downstream checkpoint activation. KSHV LANA has been shown to interact with p53 and likely modifies p53 function [69–71]. Therefore, the collective strategy of latent KSHV infection is to target the host growth-suppressive DDR by increased mutagenesis, direct binding to p53, overcoming cellular checkpoints and upregulation of antiapoptotic molecules. However, further proliferation of infected cells favors mutations in DDR genes and possibly loss of viral DNA (Figure 3A). The latter notion is supported by the presence of cells undergoing lytic replication within KS lesions that may maintain the pool of latently infected cells [66,74].

KSHV lytic infection & DDR suppression

The role of viral DNA in activating the DDR during lytic KSHV infection has not been well characterized. However, it is likely to generate similar structures and have similar consequences as EBV lytic replication (Figure 3B). Therefore, it is not surprising that the KSHV vIRF1 protein has emerged as a suppressor of DNA damage signaling [83]. The suppressive activity of vIRF1 is linked to its ability to directly interact with the DDR-sensing kinase ATM. Interestingly, similar to the EBV Zta protein, vIRF1 also interferes with p53 function, suggesting a convergent solution to the problem of DDR activation during γ -herpesvirus lytic reactivation (Figure 3B).

HPV & the DDR

Human papillomaviruses are small DNA tumor viruses that infect squamous epithelial cells and are the etiological agent of cervical cancer [84,85]. The HPV life cycle is tightly connected to infected keratinocyte differentiation. Initially, HPV infects undifferentiated keratinocytes in the basal layer of the squamous epithelium accessed through microwounding [85]. The HPV genome is maintained as circular episomes and is replicated through theta structure intermediates [86] in infected basal cells where the viral E1, E2, E6 and E7 proteins are expressed [87]. E1 and E2 replicate and maintain the viral episomes [88], while E6 and E7 promote cell proliferation through p53 and Rb antagonism, respectively [89–93]. Lytic DNA replication (referred to as vegetative DNA replication in papillomaviruses), late capsid proteins L1 and L2 expression and HPV virion assembly are limited to differentiated cells of the upper spinous layers of the squamous epithelium [85].

HPV-infected undifferentiated keratinocytes display non-canonical ATM activation

Recent evidence indicates that HPV provokes a DDR in primary human foreskin keratinocytes (HFKs) [94]. HPV early gene expression in undifferentiated primary keratinocytes leads to activation of several markers of the host ATR/Chk1 and ATM/Chk2 DDR signaling pathway including phospho-Chk1-Ser317, phospho-ATM-Ser1981, phospho-Chk2-Thr68, phospho-p53-Ser15 and γ H2AX (Figure 4A) [94]. However, ATM was not canonically activated since its upstream activator, and also key downstream target, Nbs1, was not phosphorylated in undifferentiated HPV-positive cells.

Activation of ATM may be caused by DNA DSB generated during robust host DNA replication mediated by HPV oncoproteins. HPV E7 has been shown to promote aberrant S-phase induction through E2F/Rb family complex disruption [90,91,95] and, recently, E6 and E7 were shown to induce replicative stress in undifferentiated keratinocytes [96]. As in other settings of acute oncogenic stress, collapsed replication forks also generate DSBs and activate the DDR [5]. This is consistent with activation of both the ATR/Chk1 and ATM/Chk2 pathways in HPV-positive undifferentiated HFK cells [94,96]. Interestingly, Moody and Laimins also demonstrated a physical association between high-risk HPV-31 E7 and the activated, Ser1981-phosphorylated ATM through the Rb-association motif, LXCXE [94]. In fact, E7 expression in the absence of HPV genomes or other viral proteins was sufficient to induce ATM-mediated Chk2 phosphorylation. Therefore, HPV E7 binds to activated ATM (or directly activates ATM upon association), and then the E7–pATM complex induces Chk2 phosphorylation. Furthermore, E7 expression has previously been shown to promote centrosome duplication, which could either be a consequence of these activities or could independently promote DDR activation [97]. Finally, *in vivo*, E7 transgenic mice display a greater number of head and neck carcinomas on a *fancd2*-knockout background with a compromised DDR pathway, supporting the notion of a tumor-suppressive E7-provoked DDR [98].

ATM/Chk2-dependent HPV episome amplification in differentiated keratinocytes

As HPV-infected keratinocytes in the basal and lower spinous layers of the squamous epithelium differentiate and move to the upper spinous layers, vegetative DNA replication (or episome amplification), late protein expression and virion assembly occurs. Moody and Laimins showed that, upon keratinocyte differentiation and viral episome amplification, ATM phosphorylates Nbs1 Ser343 [94]. Interestingly, ATM or Chk2 inhibition prevented episome amplification upon differentiation, but not the maintenance of HPV episomes in undifferentiated HFKs. Prior work from the same group demonstrated that low-level activation of caspase 3/7 in differentiated keratinocytes cleaved the viral E1 protein and was important for HPV genome amplification [99]. Therefore, since inhibition of ATM or Chk2

suppressed episome amplification, it was of interest to note that Chk2 inhibition also prevented HFK differentiation-induced caspase 7 activation [94]. Collectively, these data suggest a model whereby an E7-pATM complex activates Chk2 and sequential low-level activation of caspase 3/7 required for E1 cleavage, facilitating genome amplification in differentiated keratinocytes (Figure 4B) [94,99].

In addition to viral oncoproteins, DNA structures associated with the amplification of viral episomes are another possible source of DDR activation in HPV-positive cells. In undifferentiated cells HPV episomes replicate bi-directionally through theta intermediates [85,86] and possibly associate with DDR factors, although this may not achieve a threshold of signal triggering checkpoint activation. Rather, upon differentiation, the robust amplification of episomes may be sufficient to induce ATM activation, which is important for proper replication [94]. Vegetative viral DNA replication through theta intermediates likely generates nicks in the viral DNA that promote a reported switch from theta structure to a rolling circle mechanism in differentiated keratinocytes [86,100] and further accumulation of DSBs. A final possible source of HPV-induced DNA damage is the re-amplification of an integrated HPV genome [101]. Reamplification of integrated HPV DNA generates DSBs and has been shown to promote genomic instability [101]. Therefore, HPV infection activates the host DDR through multiple mechanisms. The DDR likely acts as a tumor suppressor in undifferentiated HPV-infected cells; however, ATM/Chk2 activation is required for normal vegetative DNA replication in differentiated cells.

Interactions of other human tumor viruses with the DDR

HBV & the DDR

HBV is among the major etiological factors of hepatocellular carcinoma and a *bona fide* human DNA tumor virus. HBV is a small DNA tumor virus that replicates through an RNA intermediate and encodes proteins through four open reading frames: HBV X protein (HBx); HBV surface proteins; HBV core proteins; and HBV polymerase genes [102]. HBx is a pleiotropic factor required for HBV replication, and has been implicated in numerous signaling pathways including the DDR [103]. Furthermore, HBV replication proceeds through nucleic acid intermediates that are likely substrates for DDR sensors.

Early hepatocyte infection by HBV activates the ssDNA-sensing kinase ATR

HBV-infected cells exhibit elevated levels of activated ATR kinase and activation of its downstream target Chk1 in the absence of ATM activation. Moreover, HBV infection increased survival of UV-treated (ATR-activated) cells but not γ -irradiated (ATM-activated) hepatocytes [104,105]. These data suggest that HBV replication, while activating ATR, also perturbs downstream signaling to ensure infected cell survival. Interestingly, the dual ATM/ATR antagonist theophylline decreased HBV protein and RNA levels in infected cells and decreased HBV surface antigen levels in the serum of HBV-transgenic mice suggesting an important role for ATR in HBV replication [105]. Therefore, HBV infection activates the host ATR kinase to promote replication, but alters downstream signaling.

HBV oncoprotein HBx affects ATR & p53 promoting genomic instability

HBV X protein is a multifunctional protein that promotes carcinogenesis and interacts with DDR signaling pathways in various ways (recently reviewed in [103,106]). In agreement with HBV infection data, ATR downstream signaling is modulated by HBx resulting in impaired intra-S-phase checkpoint activation and accumulated genomic instability [107,108]. Furthermore, HBx, in collaboration with HBV core protein, represses the activity of the tumor suppressor p53 through several mechanisms [109,110]. HBx directly associates with p53 and sequesters it in the cytoplasm, thereby disrupting p53 interactions critical for

transcription [111–114]. HBx also abrogates p53 sequence-specific DNA-binding activity [115]. In addition, HBx increases mutagenesis in HBV-infected cells through inactivation of proteins involved in nucleotide excision repair [116–120]. Finally, HBx interferes with centrosome replication, leading to chromosomal rearrangements and increasing the frequency of multinucleated cells [121–124]. Therefore, HBx has robust antiapoptotic activity and the consequences of its interactions with p53 and other proteins are increased levels of genomic instability in HBV-infected cells.

Polyomaviruses & the DDR

Polyomaviruses are small non-enveloped viruses containing circular dsDNA genomes of approximately 5 kb that can infect most cell types and cause tumors. The majority of our understanding regarding polyomavirus transformation comes from studies of simian virus (SV)40 and its T, or tumor, antigen [125]. However, until recently the role of polyomaviruses in human cancer was equivocal. Two well-characterized human polyomaviruses are BK virus and JC virus. JC virus causes progressive multifocal leukoencephalopathy and BK virus causes nephropathy in immunocompromised individuals. Despite much effort, however, there is little evidence to suggest that these two viruses are the etiologic agents of any human cancers [126,127]. By contrast, a third, more recently described human polyomavirus has been identified in Merkel cell carcinomas, a rare aggressive skin cancer, and is called Merkel cell polyomavirus [128]. In Merkel cell carcinomas, a truncated T antigen is expressed that is capable of Rb association similar to SV40 T antigen and HPV E7, but incapable of promoting replication [129]. Increasing evidence suggests that this will be another *bona fide* human tumor virus.

One of the first lines of evidence for the interaction between polyomaviruses and the host DDR came from the observation that SV40 large T antigen reduced protein levels of the ATM-activating MRN complex [130] and decreased MRN foci formation in response to γ -irradiation [131]. Reduced MRN protein levels in SV40-infected primate cells were found to be ubiquitin ligase CUL7-dependent and mediated through proteasome degradation [132]. Furthermore, T-antigen-mediated targeting of Nbs1 promoted both cellular endo-reduplication and replication of SV40 origin-containing DNA [133]. Moreover, polyomavirus infection activates ATM, likely through the unlicensed replication of viral DNA, and results in the induction of an intra-S-phase checkpoint with accumulation of infected cells in S and G2-phases of the cell cycle [134]. Interestingly, increased viral DNA replication depends on SMC1, but not p53, as SV40 replicates to a higher yield in ATM^{+/+} p53^{-/-} and wild-type SMC1 but not ATM^{-/-} p53^{-/-} and SMC1 mutant mouse embryonic fibroblasts [134].

Simian virus40 infection was also shown to trigger ATR-dependent Chk1 activation. Consistently, Rad51 and FancD2 co-localized with large T antigens in viral replication centers and were important for efficient viral replication [135]. A unique aspect of SV40-mediated ATR activation is the induction and phosphorylation of a splice variant of p53, Δ p53, which specifically upregulates p21 and activates an intra-S-phase checkpoint by virtue of attenuating cyclin A/cdk1/2 activity [136]. This pathway is critical for virus replication, in part due to a change in the phosphorylation of DNA polymerase α such that the hypo-phosphorylated form associates with SV40 T antigen and enables viral, but not cellular, DNA replication [136]. Therefore, SV40 polyomavirus takes advantage of the host DDR to pause the infected cell in S-phase where optimal conditions exist for viral DNA replication.

Discussion & future perspective

DNA tumor virus infections activate and modulate DNA damage-responsive signaling pathways through multiple independent mechanisms. First, viral oncoprotein-driven aberrant cellular proliferation is capable of triggering host DNA replicative stress. Second, viral oncoproteins can directly target DDR signaling pathway components during latent and lytic infection. Third, viral DNA episomes, damaged viral DNA, or exposed viral DNA ends are capable of activating the DDR during latent or productive infection.

Viral oncoprotein-driven proliferation of infected cells can be viewed simply as the propagation of viral DNA in daughter cells in the absence of virion production. DNA tumor viruses use different mechanisms to promote proliferation of the host cell. EBV EBNA2 and EBNA-LP expressed in early EBV latent infection potently upregulate *c-Myc* transcription and B-cell hyperproliferation [39]. KSHV v-cyclin directly activates cyclin-dependent kinase 6 driving S-phase [79]. HPV E7 and polyomavirus T antigens bind to Rb family members promoting E2F-mediated expression of cyclins A and E and S-phase entry [90,91,95,137,138]. However, unscheduled proliferation of host cells activates a growth-suppressive DDR [16].

To abolish the host tumor suppressive DDR pathway, DNA tumor virus proteins either attenuate or directly inhibit the host DDR. Full latency III gene expression in EBV-transformed LCLs attenuates the early-infected hyperproliferation-induced DDR [39]. By contrast, overexpression of EBV oncoproteins downregulate ATM expression (LMP-1) [48] or modulate Chk2 downstream signaling (EBNA3C) [46]. KSHV LANA has also been observed to directly bind to p53, potentially affecting its activity [69,71,139]. HPV E6 protein-mediated destabilization of p53 is sufficient to protect from an E7-driven proliferation-associated DDR [89]. In addition, recent evidence suggests that the E7:pATM interaction likely manipulates ATM downstream signaling to the benefit of HPV replication [94]. HBV HBx protein also directly antagonizes the function of p53 overriding DDR activation and likely contributing to the development of hepatocellular carcinoma [106].

During viral DNA replication, the nucleic acid structures generated are often capable of inducing the host DDR. In the case of EBV, the viral DNA replication-initiating and EBNA1 binding site, oriP, contains sequences resembling telomeric repeats [54]. These nonamer sequences associate directly or indirectly with several DNA damage and repair factors. The DSB-recognizing MRN complex, as well as TRF2 and Rap1, associate with these repeats and facilitate viral DNA replication [19,55]. By contrast, PARP1, tankyrase and TRF1 associate with oriP and disrupt the normal architecture of the replication complex [56,57]. While these proteins associate with oriP and EBNA1 and alter the fate of the EBV episome, they do not appear to activate cell cycle checkpoints in response to viral DNA replication.

Viral DNA replicating during the lytic cycle of oncogenic virus infection activates robust DDR targeting checkpoint proteins [58,94]. In these settings, however, viral lytic proteins are expressed that counteract these activities. For example, EBV Zta appears to interfere with ATM activation through interaction with 53BP1 as well as modulation of downstream p53 transcriptional activity [61,62]. Similarly, the KSHV vIRF1 protein attenuates ATM signaling and suppresses p53 likely enabling efficient KSHV lytic replication [83]. HPV episome amplification appears to require ATM signaling [94]; however, the downstream consequences of this activation have not yet been characterized. In the case of SV40, T-antigen-dependent replication also relies on ATM activation [140].

The induction of the DDR by viral DNA structures and oncoproteins is certainly an important control point during the life cycle of these viruses. However, another mechanism

by which the DDR could be activated altering the outcome of oncogenic virus infection is through acute or chronic inflammation. It is well recognized that inflammation is an important cofactor in cancer [141]. Acute virus infection that triggers an inflammatory response would lead to the accumulation of ROS and DDR activation. If activated prior to viral defense mechanisms, this response could ultimately limit viral DNA replication. Furthermore, chronic inflammation such as during persistent microbial infection or autoimmunity could lead to elevated ROS levels that may synergize with viral oncoprotein-driven mutagenesis and genomic instability. The interplay between chronic infections, such as those that lead to cancer, inflammation and the activity of DDR signaling pathways is therefore an important area for future study.

Together with innate immunity and the adaptive immune system, the DDR has emerged as a key intrinsic barrier to DNA tumor virus infection. Given the growth-suppressive nature of the DDR in the context of latent oncoprotein-driven aberrant cell proliferation, prospective therapy aimed at activating the host DDR in the early stages of viral infection would selectively induce apoptosis and potentially clear the viral infection. This concept is supported by experiments indicating that cells lacking DDR components are hypersensitive to p53 activation through antagonizing its major ubiquitin ligase, MDM2 [142]. In fact, both KSHV- and EBV-infected cells are sensitive to MDM2 antagonists called Nutlins, suggesting that a primed DDR pathway may be targeted therapeutically in KSHV- and EBV-infected tumors with intact p53 [143–145].

The discoveries over the past few years concerning the interactions between human DNA tumor viruses and the host DDR have opened many novel and interesting questions to be solved by the field. First, despite an initial characterization of the DDR signaling pathways activated by each virus, the precise source of DNA damage and which DDR sensors are recruited to these sites dynamically during virus infection remains to be determined. For example, in the case of latent EBV infection, while viral DNA has been ruled out, evidence for telomere exposure exists, as well as replicative stress. In the case of HPV, experiments with mutant genomes where the E7 interaction with ATM is perturbed will distinguish a direct DDR activation by viral oncoproteins from viral DNA or replicative stress-induced activation. Another key area of research will be to determine the mechanisms by which viral proteins attenuate the DDR during different stages of infection. For example, does EBNA3C attenuate the DDR in early proliferating EBV-infected blasts through direct attenuation of EBNA2-driven transcription of *c-Myc* or through a more complex mechanism. Perhaps EBNA3C attenuates the DDR through methylation of growth control genes, Chk2 inhibition, or by modestly perturbing p53 function. Finally, defining the source of DNA damage during lytic replication of these viruses will illuminate the role of viral proteins in controlling the cell cycle versus the intrinsic sensing of viral DNA ends prior to packaging into nascent virions. Excitingly, studies over the next several years will likely provide insights into fundamental biological problems, ranging from the intrinsic host response to DNA damage as a result of genotoxic versus oncogenic stress. It is likely that, as in the case of many key historical examples, viruses will continue to provide robust clues into normal and pathologic cell biology.

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Executive summary

The DNA damage response acts as an innate barrier to tumorigenesis

- Activated oncogenes lead to replicative stress causing ataxia-telangiectasia RAD3-related (ATR) kinase activation and, ultimately, DNA double-stranded break formation activating the ataxia-telangiectasia mutated (ATM) kinase.
- Downstream effectors of ATR and ATM signaling coordinate cell cycle checkpoints and DNA repair activities following damage.
- Mutations in DNA damage response (DDR) genes increase susceptibility to tumor development.

Human DNA tumor viruses

- EBV, Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), human papillomavirus (HPV) and HBV are human DNA tumor viruses that cause associated malignancies.
- DNA tumor viruses interact with the host DDR by the induction of replicative stress due to robust proliferation of host cells, direct binding of viral proteins to host DDR proteins and exposed structures during viral DNA replication.

EBV & the DDR

- EBV is a γ -herpesvirus that causes lymphomas, including African Burkitt's lymphoma, and epithelial cancers, such as nasopharyngeal carcinoma.
- EBV latent gene expression activates a transient ATM/Chk2-mediated growth-suppressive phenotype. A switch in viral promoter usage facilitates changes in viral early latency program that attenuate DDR activation, resulting in stable karyotypes of EBV-immortalized lymphoblastoid cell lines.
- Independent overexpression of EBNA1, EBNA3C and LMP1 in Burkitt's lymphoma cells induces genomic instability through increased levels of ROS, disruption of mitotic checkpoints, and repression of the DDR sensor ATM, respectively.
- Latent EBV episome replication requires DDR proteins, including TRF2 and RAP1. During lytic replication, viral DNA together with the major tegument protein BPLF1 activate ATM/Chk2 signaling. However, the major EBV lytic switch protein Zta interacts with p53 and 53BP1 to prevent checkpoint activation and facilitate viral DNA replication.

KSHV & the DDR

- KSHV is a γ -herpesvirus that is implicated in several human tumors including KS and AIDS-associated primary effusion lymphomas.
- KSHV infection of endothelial cells *in vitro* and early-stage (patch) KS lesions *in vivo* strongly upregulate DDR markers, while later (nodular) KS lesions displayed an attenuated DDR.
- Expression of the KSHV protein v-cyclin in primary human dermal endothelial cells induced a robust ATM/Chk2-mediated response. latency-associated nuclear antigen protein, aside from its normal role in episome maintenance, also forms a complex with p53 and, likely, modifies its function.

- During KSHV lytic replication, vIRF1 suppresses ATM by direct interaction and interferes with p53 function.

HPV & the DDR

- HPVs are small DNA tumor viruses that infect squamous epithelial cells and cause cervical cancer.
- HPV-positive undifferentiated primary keratinocytes display hallmarks of an activated ATM/Chk2-mediated DDR. This may be due to exposed viral DNA or oncoprotein expression.
- HPV E7 promotes aberrant S-phase induction through E2F/Rb family complex disruption, likely causing replicative stress in undifferentiated keratinocytes. E7 may directly promote genomic instability through centrosome duplication as well.
- E7 independently binds to activated ATM and induces Chk2 phosphorylation. This activity may be important for inducing low-level caspase 3/7 activation required for E1 cleavage and genome amplification in differentiated keratinocytes.
- HPV episome amplification requires ATM and Chk2 activity.

Interactions of other DNA tumor viruses with the DDR

- HBV infection activates the ATR sensor kinase.
- HBV X protein has robust antiapoptotic activity; HBV X interactions with p53 and other proteins increase genomic instability in HBV-infected cells.
- Polyomavirus simian virus (SV)40 DNA replication depends on DDR components ATM and SMC1, but not on p53.
- SV40 T antigen activates and perturbs DDR signaling to arrest the infected cell in S-phase, enabling virus, but not host, DNA replication. Uncoupling of these activities through viral genome insertion and mutagenesis may drive tumorigenesis.
- The newly described human Merkel cell polyomavirus may drive Merkel cell carcinoma through the uncoupling of T antigen-dependent viral DNA replication from the induction of aberrant cell proliferation and DDR activation.

Figure 1. DNA damage response to single-stranded and double-stranded breaks

(A) ssDNA is coated by RPA and subsequently recognized by an ATR–ATRIP complex, while the RAD9–RAD1–HUS1 (9–1–1) complex binds to the 5′ overhang near the exposed ssDNA. The 9–1–1 complex brings the adaptor TOPBP1 that activates ATR in the presence of ATRIP and leads to ATR-mediated phosphorylation of downstream targets including Chk1 and p53. (B) DNA double-stranded breaks are detected by the Mre11–Rad50–Nbs1 complex, which promotes monomerization and autophosphorylation of the ATM kinase at Ser1981. Activated ATM initiates a downstream cascade of events, including phosphorylation and activation of its downstream effector Chk2 kinase at Thr-68, p53 at Ser-15, H2AX tail at Ser-139 (γH2AX) and Nbs1 at Ser343, as well as the phosphorylation of more than 180 targets. The phosphorylation of H2AX serves as a scaffold for the recruitment of MDC1 (not shown) and 53BP1 (among other proteins) at the site of the dsDNA breaks, enabling signal amplification and propagation.

ATM: Ataxia-telangiectasia mutated kinase; ATR: Ataxia-telangiectasia and RAD3-related kinase; ATRIP: Ataxia-telangiectasia and RAD3-related kinase-interacting protein; RPA: Replication protein A; TOPBP: Topoisomerase binding protein.

Figure 2. EBV infection activates the host DNA damage response

(A) EBV infection of human primary B lymphocytes *in vitro* initiates EBNA2 and EBNA-LP-mediated c-Myc expression leading to cellular hyperproliferation and activation of an ATM/Chk2-dependent DNA damage response (DDR). Importantly, DDR foci are not colocalized with viral episomes. As infected cells proliferate five to six times, expression of the essential viral oncoproteins EBNA3C and LMP1 (among others) attenuates cellular proliferation leading to downregulation of the host DDR and growth transformation of infected cells into lymphoblastoid cell lines. (B) Transfection of individual viral latency proteins in BL-derived cells modifies DDR signaling. LMP1 expression represses ATM expression; EBNA3C abolishes the mitotic spindle checkpoint through downregulation of BubR1 and leads to EBNA3C:Chk2 complex formation; whereas EBNA1 expression, through induction of ROS, activates ATM and leads to phosphorylation of H2AX. (C) Lytic EBV viral DNA replication generates double-stranded breaks (shown as sparks) that activate ATM downstream signaling. The EBV major tegument protein BPLF1 induces cellular DNA hyper-replication through stabilization of the licensing factor Cdt1. The consequent unchecked S-phase activates an ATR- (not shown) and ATM-dependent DDR. However, viral lytic protein Zta, or BZLF1, binds to 53BP1, promoting the lytic cycle and also inhibits p53 through multiple mechanisms.

ATM: Ataxia-telangiectasia mutated kinase; BL: Burkitt's lymphoma; EBNA: EBV nuclear antigen; LMP: Latent membrane protein; ROS: Reactive oxygen species.

Figure 3. Kaposi's sarcoma virus targets the host DNA damage response

(A) KSHV latently infected endothelial cells within early KS lesions display an activated ATM/Chk2-mediated DDR, likely mediated through v-cyclin-driven cellular proliferation, centrosome duplication and LANA targeting of growth suppressive pathways. Later, KSHV latent infection is characterized by attenuated ATM/Chk2 signaling, which may be a result of selection of mutated DDR pathway components, disruption of cellular checkpoints, and LANA binding to p53. (B) KSHV lytic replication likely activates ATM, similarly to Figure 2C. To promote KSHV replication, the viral lytic product vIRF1 interacts with ATM and manipulates p53 function.

ATM: Ataxia-telangiectasia mutated kinase; DDR: DNA damage response; KS: Kaposi's sarcoma; KSHV: Kaposi's sarcoma-associated herpesvirus; LANA: Latency-associated nuclear antigen.

Figure 4. Human papillomavirus manipulates the host ataxia-telangiectasia mutated signaling (A) Basal epithelial cell infection by HPV (left) leads to expression of viral E1, E2, E6 and E7 proteins, which activate ATM/Chk2 and ataxia-telangiectasia and RAD3-related kinase/Chk1 (not shown) signaling (middle). Episome DNA replication through a theta intermediate likely maintains the HPV genome in infected undifferentiated keratinocytes. In Ca²⁺-differentiated keratinocytes (right), where viral episome amplification occurs through a possible rolling circle mechanism, Nbs1 is also phosphorylated and ATM and Chk2 are important for viral DNA replication. The E7 protein binds to activated ATM (or activates ATM through binding) and manipulates ATM signaling (B). (B) Putative mechanism of caspase-cleaved E1-dependent genome amplification in differentiated cells. The HPV E7:pATM complex (or HPV DNA) leads to modest activation (dashed arrow) of caspases 3 and/or 7, not inducing apoptosis, but resulting in E1 cleavage, which is required for HPV genome amplification.

ATM: Ataxia-telangiectasia mutated kinase; HPV: Human papillomavirus.