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Manipulation of Cellular DNA Damage Repair Machinery Facilitates Propagation of Human Papillomaviruses

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Viruses and DNA Damage Repair

Because cellular DNA is constantly bombarded by exposure to endogenous and exogenous mutagens; a vast network of proteins, collectively referred to as DNA Damage Repair (DDR) machinery, has evolved in response to these insults. DDR pathways are capable of sensing damaged DNA, inducing a signaling cascade, and ultimately recruiting the DDR-specific nucleases, helicases, ligases, and polymerases necessary to pause cell cycle progression and repair the resulting lesions. Impressively, DDR pathways repair an estimated 10,000 lesions per cell per day [1].

Although there is some interplay between the pathways, specific repair pathways are generally dedicated to the repair of particular types of damaged DNA. As an overview, Figure 1 depicts three common forms of damaged DNA and some of the proteins/pathways that are activated in response to these lesions. For example, while the PI3 kinase ATM is activated by phosphorylation in response to double strand breaks in DNA (DSBs), a related PI3 kinase ATR and its interacting partner ATRIP respond to intrastrand crosslinks in DNA (Figure 1). The activation of these kinases results in the initiation of downstream repair pathways. Similarly, in response to single strand breaks in DNA (SSBs), PARP1 and the MRN complex initiate repair of the lesion. Together, DNA repair pathways maintain the fidelity of the human genome.

The importance of DDR proteins is not limited to protecting genomic material from these numerous insults. Indeed, many DDR proteins also play a role in the body's immune response. A subset of DDR proteins participate in the recombination of antibody genes that results in our almost unlimited diversity of antibody response [2]. Additionally, many of these same proteins play a role both in the cellular response to viral infections as well as the lifecycle of multiple viruses. In general, the interplay among viruses and DDR proteins can

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be divided based on whether the interaction inhibits or promotes viral propagation. In some cases, such as Adenovirus infections, host DDR pathways act to restrict viral propagation [3–5]. Predictably, many viruses that are adversely affected by host DDR machinery have evolved means to subvert the DDR response [6–14]. On the opposite end of the spectrum, other viruses, such as members of the herpesvirus, polyomavirus and papillomavirus families, rely on the host DDR response to replicate their genomes. This viral strategy involves the activation of DDR proteins and their recruitment to viral replication centers, providing viral replication centers access to DDR-associated polymerases that are independent of origin licensing requirements [15–27].

While many viruses exclusively employ one or the other of these two strategies, some viruses like the human papillomaviruses (HPVs) have more complicated relationship with host DDR pathways. HPVs both inhibit and activate different aspects of these pathways. What may seem like a paradoxical strategy is believed to allow the virus access to DDR proteins that facilitate replication of the viral genome while avoiding the cell cycle arrest that typically accompanies DDR activation. In this review, the relationship between HPV propagation and host cell DNA damage repair will be explored.

Brief Introduction on Human Papillomavirus

Human papillomaviruses (HPVs) are a large family of double strand DNA viruses that infect the mucus membranes and epidermis of humans. Although there are approximately 200 different types of HPVs divided among 5 genera, the most clinically relevant HPVs belong to the alpha-papilloma genus. As a result, most of the research on HPV proteins focuses on members of this genus; particularly those HPVs most closely connected with anogenital track cancers. Consequently, this review will focus primarily on interactions among cancer-associated alpha-papillomaviral proteins and cellular DDR proteins. However, although this review will concentrate on these particular HPVs, we will also highlight some key observations about other members of this family that help illustrate the common need for disrupting certain DDR responses.

Human Papillomavirus and the DNA Damage Response

DDR is both inhibitory and necessary for the replication of HPVs and as a result HPV proteins both activate and inhibit DDR responses. Similar to the examples discussed above, HPV proteins, particularly HPV E1 and E2, stimulate DDR at sites of viral replication, most likely in order to allow the replication centers access to cellular replication machinery [28, 29]. In response to damaged DNA, cell cycle progression is halted to allow time for repair of the damage to take place prior to attempting synthesis of new DNA from a faulty template. Because HPV replication can only occur in actively cycling cells, the viral E7 protein has evolved to push cells into a proliferative state despite contradictory signals, such as those elicited by HPV E1 and E2 induced activation of the DDR response. The ability of HPV E7 to drive cells through the cell cycle can have detrimental consequences, namely large scale genomic instability and damage. These insults to a cell’s genomic material would normally lead to apoptosis. HPV E6, however, increases cellular tolerance of DNA damage by decoupling DDR signaling from apoptotic signaling. HPV E6, also, promotes continued

advancement the cell cycle by directly inhibiting multiple DDR pathways. This review will discuss the stimulation and restriction of DDR response by HPV proteins in further detail (For a more general discussion of DDR and viruses see Lilley et al 2007 [30])

A Brief Overview of the HPV Lifecycle

The HPV lifecycle is tied to epithelial differentiation and can be divided into two phases, genome maintenance and genome amplification, based on markedly different replication strategies. Because the demands of these proliferative tactics differ widely, the interactions among viral proteins and cellular DDR proteins also vary greatly. To frame the later discussions, we will first briefly outline the replicative lifecycle of HPV, specifically highlighting the roles of viral and cellular DDR proteins. For a more thorough review of the HPV viral life cycle see Doorbar 2005 [31].

HPV Genome Maintenance

In the genome maintenance portion of the lifecycle, the viral episome is sustained at a steady copy number in the basal epithelium and viral replication is linked to cell cycle progression [31]. Viral genome maintenance requires both cellular replication machinery and at least the viral E1 and E2 proteins (some viruses also require HPV E7). While the role of DDR proteins in viral replication has not been completely elucidated, expression of HPV E1 activates the cellular DDR response and most of proteins activated in this response are mislocalized to centers of viral replication. Figure 1A depicts the HPV genome tethered to the host cell's genome by an interaction involving TOPBP1 as well as HPV E2. Additionally, as can be seen in the figure, the HPV genome has been shown to colocalize with multiple DDR proteins, including members of the ATM, ATR, and homologous recombination pathways (Figure 1A). (The relationship between HPV E1, as well as HPV E2, and cellular DDR is covered in greater detail in a later section.)

HPV Genome Amplification

HPV amplification is the differentiation-dependent portion of the viral lifecycle when infectious virions are produced. During amplification, the copy number of HPV viral genomes per cell is greatly increased and they are packaged into equally numerous viral particles [31]. All HPV proteins are expressed during amplification, including HPV E1, HPV E2, HPV E6 and HPV E7. Higher viral copy number results in greater HPV E1 and E2 expression and in a corresponding increase in DDR stimulation as well as the associated intensification of anti-proliferative signals. During amplification, the increased DDR response associated with viral replication centers is manifested by enlarged DDR/viral replication foci as depicted in Figure 1B. Additionally, amplification occurs in cells that would typically have exited the cell cycle as part of epithelial differentiation. HPV E7 helps drive these normally quiescent cells into the proliferative state required for HPV replication. As previously discussed, HPV E7-mediated unbridled replication results in large scale damage to the cellular genomic. Requiring, HPV E6 to increase the tolerance of this damage and allow cells to avoid apoptosis and continue proliferating. (The relationship between HPV E6 and HPV E7 and the cellular DDR response is discussed in greater detail in subsequent sections.)

Cellular DDR response and HPV E1 and E2

The replication of HPV genome is dependent on the viral proteins, HPV E1 and E2. HPV E2 simultaneously binds the viral origin of replication and HPV E1. This allows HPV E2 to recruit several HPV E1 monomers to the origin, where a double hexameric HPV E1 complex is formed. The HPV E1 double hexamer functions as a helicase, unwinding viral DNA, and together with cellular proteins, replicates the viral genome. (For a more generalized review of HPV replication, see Stenlund 2003 [32].)

HPV E1 and DNA Damage Repair Proteins

HPV E1 consists of three functional domains. Many regulatory and localization signals are found in the N-terminus of the protein, including a nuclear localization signal and nuclear export signal as well as cyclin E/A binding motif and two Cdk2 phosphorylation sites [33, 34]. The central region of the protein contains the origin-binding domain that along with HPV E2, is required for binding the viral origin of replication. The most functionally distinct domain of HPV E1 is its' helicase domain, located in C-terminus of the protein. Each of these domains and sites is required for efficient viral replication [33, 34].

Multiple groups have shown that the expression of HPV E1 can activate a DNA damage response and induce an S-phase arrest (Table 1, Table 2) [28, 29, 35]. This activation is dependent on origin binding and helicase domains of HPV E1 and involves the auto-phosphorylation of the DDR kinase, Ataxia Telangiectasia Mutated (ATM). Typically, ATM becomes activated through auto-phosphorylation primarily in response to double strand breaks in DNA or DSBs [36]. Once activated ATM then phosphorylates a multitude of targets including the histone variant H2AX and the ATM-effector kinase Chk2. This begins a signaling cascade that triggers repair of the lesion as well as cell cycle arrest. Indicative of viral-induced ATM activation, not only is ATM activated by HPV E1 expression, but Chk2 and H2AX are phosphorylated and cells arrest in early S-phase.

Similarly to ATM, the DDR kinase, ATM and Rad3-related (ATR), once activated, initiates a series of phosphorylation events resulting in the repair of DNA lesions. However, unlike ATM, ATR is primarily activated in response to replication stress instead of DSBs [37]. Perhaps not surprisingly given their similarities, ATR is also activated in response to HPV E1 expression, resulting in the phosphorylation of both ATR and Chk1, a downstream target of ATR. Interestingly, unlike ATM, ATR is only activated in a subset of HPV E1 expressing cells suggesting that this activation occurs during certain phases of the cell cycle [29].

The activation of the ATM pathway by HPV E1 may be the result of non-specific E1 helicase activity as the E1 helicase has been reported to have some non-specific activity[28]. The activation of the DDR is dependent on the nuclear accumulation of E1 and both COMET assay as well as *in situ* TUNEL staining confirm that damaged DNA is leading to the activation of the ATM pathway [29, 35]. Interestingly, HPV E2 expression can attenuate DDR activation by reducing the accumulation of HPV E1 in the nucleus, however HPV E1-associated cell cycle arrest still occurs. The mechanism by which HPV E1 induces cell cycle arrest independent of its activation of ATM has not yet been elucidated. Nevertheless, the

ability of HPV E1 to halt the cell cycle progression in S-phase could be advantageous for HPV, by increasing the availability of the cellular replication apparatus.

HPV E2 and DNA Damage Repair Proteins

HPV E2 is a modular protein, composed of a C-terminal DNA-binding domain and an N-terminal transactivation domain. The HPV E2 protein plays several roles in the viral lifecycle that can be divided between activities important for viral replication and those important for viral transcription. The C-terminal domain of HPV E2 binds the HPV genome and tethers it to the host genome thus insuring the faithful segregation of viral progeny during mitosis. Furthermore, HPV E2 helps form the viral replication complex and stimulates replication initiation in these complexes. Further, it insures the faithful segregation of viral episomes into daughter cells during cytokinesis by tethering the viral and host genome through an interaction with the cellular DDR protein TopBP1 (Fig 1A, Table 1) [38–40].

HPV E2 is also a major regulator of viral gene expression through its interaction with p300 and p300/CBP-Associated Factor (p/CAF), two cellular histone acetyl transferases (HATs) [41, 42]. Together with these HATs, HPV E2 promotes the hyperacetylation of the viral early promoter leading to the expression of HPV E6 and E7 [42, 43]. p300 and p/CAF also play central roles in the DDR. Following DNA damage, p300 and p/CAF acetylate p53 [44, 45]. The ability of HPV E2 to utilize both p300 and p/CAF to drive viral transcription suggests the possibility that HPV E2 may attenuate the ability of these HATs in response to DNA damage.

Following DNA damage, p300 and p/CAF acetylate p53 increasing p53's sequence specific binding capability and thus its ability to drive target gene transcription, ultimately resulting in a fully activated repair response [46]. Additionally, recent work further links p300 to the p53-DDR as p300 is required for robust expression of both ATM and ATR [47, 48]. These kinases prevent the proteosomal degradation of p53 by phosphorylating it in response to DNA damage [49, 50]. When fully activated by phosphorylation and acetylation, p53 efficiently drives transcription of its target genes, resulting in apoptosis or cell cycle arrest in order to facilitate DDR. In addition to potentially interfering with p53 activation indirectly, HPV E2 may interfere with p53 activation through a direct interaction with the tumor suppressor [51]. If HPV E2 is capable of attenuating p53 activation, the resulting attenuated DDR combined with HPV E2's ability to directly activate caspase 8 [52] may explain the p53-dependent induction of apoptosis by HPV E2 expression (Table 2) [53].

Viral Replication Complexes and DNA Repair Proteins

HPV E1 and E2 proteins, as well as the viral genome, form the core of the viral replication complex. HPV E1 and E2 colocalize together in nuclear foci, where they facilitate replication of the viral genome [35]. *In vitro* studies have shown that although both HPV E1 and E2 are required for initiation of viral genome replication, only HPV E1 is necessary for the elongation [54]. The details of how E1 and E2 stimulate viral genome replication are not fully understood, but DDR proteins are likely heavily involved in the process. A multitude of cellular DDR proteins colocalize with the HPV replication complex, including ATRip,

TOPbp1, phospho-ATM, phospho-H2AX, phospho-p53, Chk1, Chk2, PCNA, RPA, Nbs1, 53bp1, BRCA1, and RAD51 (Fig 1A, Table 1) [29, 35, 55]. Interestingly, as discussed in a previous section, non-specific HPV E1 helicase activity is believed to induce the activation of many of the DNA damage proteins that are also colocalized to centers of viral replication. This suggests that the non-specific nature of HPV E1 helicase allows the virus to induce a DDR by damaging the host genome and that the responding DDR proteins are usurped to promote the replication of the viral genome.

Although numerous repair proteins support viral replication, the Fanconi Anemia (FA) repair pathway restricts HPV genome amplification. The FA repair pathway is named for the rare genetic disorder caused by disruption of the pathway [56]. FA repair responds to damage caused by replication stress as well as cross-linked DNA and consists of two protein complexes, the core complex that contains the FA pathway proteins FANCA, B, C, E, F, G, L, M, FAAP24, AND FAAP100, as well as the ID complex that contains both FANCD2 and FANCI. When activated, the core complex ubiquitinates both FANCD2 and FANCI resulting in FANCD2 foci formation, cell cycle arrest and the repair of the damaged DNA. Viral genome amplification is enhanced by the knockdown of essential components of either the FA Core or ID complex. Similar disruption of either complex also increases epithelial proliferation [57, 58]. Together these observations may explain the observation that FA patients are at a greater risk for HPV-associated Head and Neck Cancers [59, 60]. Indeed, in a mouse model, FANCD2 −/− were more sensitive to HPV-associated Head and Neck Cancers [61].

The importance of DDR proteins in facilitating HPV replication is magnified when HPV replication shifts from genome maintenance to genome amplification. This transition marks a striking increase in replication, when viral genomes increase from an estimated 10–200 copies per cell to 1000’s of copies per cell [31]. The size of DDR foci associated with viral replication centers is reported to increase during genome amplification [55]. Furthermore, the HPV-induced activation of the ATM pathway and of Caspase 3, 7, and 9 are required for viral genome amplification, but not for genome maintenance [62, 63] (Table 2). Activated caspase cleaves HPV E1 protein, potentially stabilizing the protein through the removal of a regulatory subunit in its N-terminal domain. This may result in an enhanced ability to promote viral genome replication that is not necessary during genome maintenance as a mutation of the caspase cleavage site attenuates HPV amplification [62].

HPV Oncogenes and DNA Damage Repair

HPV E6 and E7 are the primary viral oncogenes. They are both transcribed from the same viral promoter and are expressed in the greatest abundance during viral amplification. Amplification is dependent on the differentiation of keratinocytes, however keratinocytes stop replicating once they differentiate. As both cellular proliferation and differentiation are requirements of viral replication, HPV E7 is needed to uncouple keratinocyte differentiation from cell cycle exit. As a consequence of the resulting uncontrolled replication as well as increased viral genome replication, a robust DDR response is elicited. In order to prevent cells from exiting the cell cycle or undergoing apoptosis in response to this activation of DDR pathways, HPV E6 expression is required. This section of the review will focus on the

interplay among these HPV oncogenes and DDR proteins. For a more complete review of HPV E6 and HPV E7, see Howie et al 2008 as well as Mclaughlin-Durbin and Munger 2009, respectively [64, 65].

DNA Damage Repair and HPV E7

HPV E7 is a small acidic protein made up of roughly 100 amino acids. It shares sequence homology with other viral proteins, namely the adenovirus E1A protein and SV40 large T antigen. Although the viral protein has an impressive variety of activities, this review will focus on those functions of HPV E7 most likely to impact DDR, specifically deregulation of cell cycle arrest and alteration of DDR signaling.

HPV E7 and Cell Cycle Deregulation

Progression through the G1/S boundary is regulated through the interactions of a family of pocket proteins (pRB, p107, and p130) with their cognate family of E2F transcription factors. Specifically, the association of hypophosphorylated pocket family proteins with E2F transcription factors prevents them from activating transcription of the genes necessary to promote the G1 to S phase transition. Hyper-phosphorylation of pocket proteins causes them to dissociate from E2F proteins, freeing these transcription factors to drive cell cycle progression (Reviewed in Frolov et al 2004[66]). HPV E7 associates with each of these pocket proteins through the LXCXE motif located in N-terminal half of the protein [67–69], an interaction that is strengthened by sequences in the C-terminus of HPV E7 [70, 71]. Through this association, HPV E7 is able to dissociate the E2F/RB complex and drive advancement through the cell cycle [72].

The association between HPV E7 and pRB is conserved among both tumorigenic (high risk) and non-tumorigenic (low risk) HPV viruses, underscoring the importance of this interaction to the viral lifecycle (Table 2). Despite the shared association, High risk (HR) HPV E7 has a stronger affinity for pRB than low risk (LR) HPV E7 [73, 74]. The strength of the HPV E7/pRB association correlates with the ability to disrupt the E2F/pRB complex [71]. In addition to higher pRB affinity and unlike LR HPV E7, HR HPV E7 also can destabilize pRB in a Cullin2 and proteosome dependent manner [75–78]. Furthermore, HR HPV promotes the degradation of both p107 and p130 [79]. Although LR HPV E7 binds pRB with lower affinity and cannot promote pRB destabilization, LR HPV E7 binds and promotes p130 degradation [80]. Interestingly, a single amino acid difference between HR and LR can explain the differential pocket family binding capabilities of the HPV E7 proteins [81, 82].

To ensure successful alteration of key cellular responses, HPVs often employ redundant manipulations of the same pathway. Characteristic of this approach are the numerous strategies employed by HPVs to ensure continued cellular proliferation without regard to differentiation state or the presence of damaged DNA. For instance, HPV E7 binds and activates multiple E2F transcription factors independent of its destabilization of pRB [83, 84]. HPV E7 also disrupts regulation of cell cycle by cyclin dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CKIs) (Table 2). CDKs and CKIs act as powerful regulators of cellular proliferation, by promoting and restricting cell cycle, respectively.

HPV E7 inhibits the activity of two CKIs, p21CIP1 and p27KIP1, the former despite elevated protein levels [85–92]. HPV E7 also promotes cell cycle progression by increasing the activity of the CDK2 complex. Activation of the CDK2 complex drives G1/S progression and is regulated by two regulatory subunits, cyclin A and E. HPV E7 activates the CDK2 complex by associating with CDK2, cyclin A and cyclin E [93–95]. In addition to directly activating CDK complexes, HPV E7 further deregulates cell cycle by stabilizing a phosphatase, CDC25A, that promotes CDK activity [96].

HPV E7 and Histone Acetylation/Deacetylation

Histone acetylation is regulated by HATs as well as histone deacetylases (HDACs) [97, 98]. Because acetylation is a powerful determinant of gene expression, including genes responsible for DDR and cell cycle arrest, it is no surprise that multiple HPV proteins targeting these histone modifiers. Indeed, the zinc finger domain of HPV E7 can bind to class I HDACs. This interaction occurs independently of the viral protein's ability to bind pRB and is believed to be mediated by the HDAC component Mi2β [98, 99]. The binding of HPV E7 to class I HDACs blocks their binding to the promoter of E2F2, a transcription factor that promotes G1/S transition. This results in increased E2F2 protein, and may contribute to the ability of HPV-infected cells to continue proliferating despite damaged DNA [98].

HPV E7 also interacts with two HATs that are involved in DNA damage repair, namely p300 and p/CAF, as well as steroid receptor coactivator-1 (SRC-1), which can recruit both p300 and p/CAF to promoters [41, 100–104]. HPV E7 binding of p/CAF requires a functional HPV E7 zinc finger domain and represses p/CAF HAT activity [100, 102]. The association of HPV E7 with SRC-1 causes SRC-1 to become mislocalized and lose its HAT activity. The combined inhibition of SRC-1, p300 and p/CAF may synergistically blunt their activation of p53 activation in response to damaged DNA. The interactions of HPV E7 with both HATs and HDACs represent a further redundancy by the viral protein that helps insure its ability to drive cell cycle progression.

HPV E7 and p53

As previously discussed, p53 plays a central role in eliciting DDR as well as cell cycle arrest in response to damaged DNA. Pausing cell cycle progression to repair damaged DNA or in response to other stimuli is detrimental to HPV proliferation. Given the propensity for multiple HPV proteins to duplicitously target the key regulatory pathways, it is not surprising that, similarly to histone acetylation, p53 is also the target of multiple HPV proteins. Interestingly, p53 protein levels are increased in HPV E7 expressing cells as a result of increased p53 stability, potentially through the inhibition of MDM2-mediated p53 degradation [76, 77, 105–108]. Furthermore, HPV E7-induced stabilization of p53 can cause apoptosis (Table 2) [76].

However, this stabilization of p53 may not fully activate the protein as HPV E7 cells can be immortalized with wildtype p53, and some p53-responsive genes involved in DDR are not activated [88, 105, 109]. Indeed, HPV E7 interferes with p53 mediated G1 arrest in response to DNA damage (Table 2). HPV E7 expressing cells have a reduced G1 arrest in response to

actinomycin D or exposure to gamma radiation [77, 110]. Instead, HPV E7 drives cells through the cell cycle in the presence of damaged DNA or artificially activated p53 [111, 112]. Similarly, HPV E7 mitigates ATR mediated cell cycle arrest in response to replicative stress. In response to a slowed or stalled replication fork, the activation of Chk1 by ATR pauses cell cycle progression to allow resolution of delayed replication fork time[113]. This activation of Chk1 is mediated by Claspin [114]. HPV E7 increases expression of the SCFβ-^{TrCP} based machinery responsible for turnover of Claspin, accelerating the proteolytic degradation of Claspin, and allowing multiple road blocks to cell cycle progression to be bypassed [115].

HPV E7 and Genomic Instability

The primary role of HPV E7 in the viral lifecycle is to deregulate cell cycle progression. However, one reason cellular proliferation, particularly mitosis, is typically tightly regulated is to prevent large scale genomic destabilization. Any erroneous step in the process would typically cause a cell to pause and either attempt to fix the mistake or commit itself to apoptosis. By disrupting this carefully regimented process, HPV E7 expression leads to a dramatic destabilization of the cellular genome (Table 1). For example, HPV E7 expression allows continued cell cycle progression in the face of anaphase bridges, chromosome misalignment and multipolar mitosis [116]. The ability of HPV E7 to delocalize dynein, a microtubule motor and component of the mitotic apparatus, correlates with the ability of the viral protein to induce chromosome alignment defects [117]. This is dependent on HPV E7's association with NuMa, a component of the nuclear mitotic apparatus that stabilizes microtubule ends and tethers them to centrosomes [117–119]. The delocalization of dynein appears to contribute to viral maintenance and amplification by disrupting the cellular differentiation programing [118]. In addition to promoting continued cycling despite a wide variety of mitotic errors, HPV E7 expression can also induce rereplication leading to polyploidy [120]. Indeed, the induction of mitotic errors combined with the deregulation of cell cycle by HPV E7 results in numerous chromosomal abnormalities including increased ploidy and chromosome loss/duplication [121, 122].

The HPV E7-mediated loss of mitotic regulation also results in aberrant centrosome duplication. Expression of the viral oncogene induces an increase in number of centrosomes by uncoupling centriole synthesis from cell division, allowing multiple daughter centrioles to form from a single maternal template [123, 124]. This loss of controlled centrosome duplication is at least partially independently of E7-mediated pRB reduction [125, 126]. Instead, HPV E7 induces centrosome duplication by associating with gamma tubulin and alters the protein's recruitment to centrosomes [126]. Additionally, unlike typical centrosome duplication, HPV E7-induced centrosome overduplication involves the activation of CDK2 complex, including CDK2, Cyclin A and Cyclin E. HPV E7 expression does not result in centrosome defects in the presence of CDK2 inhibitor or in cells with CDK2 levels reduced by RNAi-mediated knockdown [127, 128]. Importantly, the extensive genomic instability induced by HPV E7 expression occurs after centrosome overduplication, suggesting that an abnormal number of centrosomes likely causes a portion of this destabilization [129].

HPV E7 and the FA pathway

Considering the amount of large scale genomic damage that HPV E7 can induce, it is not surprising that expression of the viral protein also activates the DNA damage response (Table 1). Most notably HPV E7 activates the Fanconi Anemia repair pathway, increasing the recruitment of both FANCD2 and BRCA2 to chromatin [130]. This activation is likely in response to HPV E7-induced DNA damage as expression of HPV E7 in a FANCD2 −/− background results in increased DSB markers, phospho-H2AX and 53bp1 [61, 130].

Not all induction of DDR by HPV E7 is solely in response to HPV E7 induced damage. HPV E7 also extends telomeres through telomerase-independent activation of the Alternate Lengthening of Telomeres (ALT) pathway [131]. The activation of the ALT-pathway by HPV E7 results in the formation of ALT-associated PML bodies that contain several DDR proteins including FANCD2, BRCA2, and Mus81. Finally, HPV E7 interacts with BRCA1, a component of the FA repair pathway and inhibits its transcriptional activation [132]. Perhaps this inhibition helps to counteract the anti-proliferative signals resulting from the cellular response to HPV E7-induced genomic instability.

HPV E7 induces Cell Death

Although HPV E7 is capable of driving cell cycle progression in the face multiple signals to halt its advancement (DNA damage and differentiation), there are deleterious effects of this unregulated proliferation. Indeed, the destabilization of pRB combined with the stabilization of p53 by HPV E7 triggers apoptosis (Table 2) [76, 133, 134]. HPV E7-induced apoptosis occurs through the activation of Chk2 and also requires both ATM and Nbs1 and is most pronounced in serum starved conditions [134]. In reaction to HPV E7 driving progression through the cell cycle in absence of growth factors, cells are more likely to undergo apoptosis [76, 135]. This predisposition to apoptose absent growth factors is known as a “trophic sentinel response” and is also seen when other oncogenes are expressed, such as adenovirus E1A (Table 2) [136, 137]. In the case of HPV E7 expression, the sensitivity to loss of growth factors is p53-dependent [135]. Finally, in addition to inducing p53-dependent apoptosis, HPV E7 expression also induces markers of autophagy, highlighting the generally deleterious result of HPV E7 expression [138].

HPV E6 and DNA Damage Response

Expression of multiple HPV proteins can activate the cellular DDR response. Once activated this response will pause the cell cycle and facilitate the repair of the damage. However, HPV E7 expression largely abrogates the cells ability to suspend cell cycle progression and as a result the extent of the damage is magnified. Extensive DNA damage typically results in apoptosis, but HPV E6 prevents DDR/DDR signaling and as a result increases cellular tolerance of DNA lesions. As is true for most HPV proteins, HPV E6 is capable of performing a wide variety functions. However, this review will highlight the roles of HPV E6 that pertain to disrupting DDR/DDR signaling.

HPV E6 and p53

Probably the most well characterized function of HPV E6 is its ability to prevent the typical cellular response to DNA damage or unlicensed replication by promoting p53 degradation (Table 1) [139, 140]. HPV E6 forms a complex with the cellular E3 ubiquitin ligase, E6AP, that ubiquitinates p53 targeting it to the proteosome for degradation [139, 141, 142]. HPV E6 binding to the N-terminus of E6AP is required for the ubiquitin ligase to target p53 [143]. Interestingly, while promoting p53 degradation is characteristic of HR HPV E6, LR HPV E6s are capable of binding E6AP, but not inducing it to ubiquitinate p53 [144]. Indeed, a recent large scale screen found that all oncogenic or potentially oncogenic HPV E6s could promote p53 degradation, while p53 levels were unperturbed by expression of any low risk HPV E6s [145]. Finally, in addition to this E6AP-dependent degradation, HPV E6 can also promote the degradation of p53 in an E6ap independent manner [146].

Underlining the necessity for HPV to avoid activation of p53, HPV E6 not only promotes p53 degradation, but also independently inhibits any remaining p53 from transactivating its target genes in response to DNA damage [147]. The domains responsible for inhibition of transactivation and p53 binding are found in functionally distinct portions of the viral oncogene [148, 149]. Notably even the inactivation of p53 transactivation occurs in a redundant manner. Not only can HPV E6 can diminish the DNA binding capability of p53 by binding the tumor suppressor but degradation-independent binding can also act to mask p53's nuclear localization signal causing it to become sequestered in the cytoplasm [149–151].

Furthermore, HPV E6 also blocks p53 transactivation indirectly by inhibiting proteins that activate p53 in *trans*. In response to DNA damage, p53's transactivation activity is enhanced by the acetylation of p53 that increases p53's DNA binding affinity [152]. HPV E6 binds three regions of the closely related HATs and inhibits p53 acetylation and transactivation [149, 153, 154]. Impressively, there are further redundancies in HPV E6's inhibition of p53 transactivation. HPV E6, also, promotes the proteosome dependent degradation of the p53 co-activator, Ada3 [155]. Ada3 complexes with p300 and p53 and, likely, acts as an adapter, facilitating the acetylation of p53 by p300 [156, 157]. HPV E6 further undermines damage-induced transactivation of p53 by delaying the activation of ATR, one of the kinases responsible for stabilizing p53 following DNA damage [47]. Underscoring the efficiency of HPV E6 inhibition of p53 transactivation, HPV E6 mutants that are incapable of degrading p53 retain their transformative potential [158].

Finally, the attenuation of the p53 by HPV E6 is shared with other members of the papillomavirus family. The E6 proteins from multiple members of the beta genus of papillomavirus also reduce p53 signaling, albeit through a distinct mechanism [47, 48, 159]. β-HPV 5 and 8 E6 bind and destabilize p300 leading to a reduction in both ATM and ATR as well as p300 protein levels. Ultimately, the diminished abundance of these key p53 modifying proteins abrogates p53 stabilization and activation in response to DNA damage, allowing cells to continue proliferating despite the presence of damaged DNA.

HPV E6 inhibits DDR

In addition to damage arising from normal cellular processes, HPV infected cells also face the induction of the DDR response by multiple HPV protein. To avoid the cessation of cell cycle progression that typically accompanies DDR, HPV E6 not only stops p53 activation but also directly disrupts multiple repair pathways insuring that they do not pause cell cycle or induce apoptosis following DNA damage (Table 1). For instance, HPV E6 attenuates the repair of single strand DNA breaks (SSB) through interactions with two proteins (XRCC1 and O^6methylguanine-DNA methyl-transferase) involved in SSB repair [160, 161]. HPV E6 expression also leads to a diminished ability to repair DSBs. While the exact mechanism for the disruption of DSB repair has not yet been elucidated, HPV E6 does interact with two proteins, BARD1 and BRCA1, linked to the homology mediated repair of DSBs (Table 1) [132, 162]. These interactions may attenuate homology mediated repair of DSBs as the majority of DSBs are repaired though homology-independent pathways in HPV E6 expressing cells [163]. Furthermore, HPV E6 impairs the repair of crosslinked DNA by blunting p53 activation in response to the damage, resulting in increased sensitivity to multiple crosslinking agents [47, 164, 165]. Attenuation of such a wide range of DDR pathways, makes HPV E6 expressing cells far more prone to mutation. Indeed, HPV E6 expression increases mutation rate in response to both endogenous and exogenous DNA damage [166].

In addition to directly inhibiting DDR and in yet another instance of overlapping functions between HPV proteins, HPV E6, like HPV E7, stimulates cell cycle progression by disrupting pRB-E2F complexes. Unlike HPV E7, HPV E6 does not destabilize pRB to deregulate cell cycle checkpoints, but instead disrupts pRB-E2F complexes by inducing the phosphorylation of pRB [167]. Furthermore, HPV E6 expression increases cellular proliferation under conditions that would typically induce cell cycle arrest. Specifically, HPV E6 expression reduces G1-arrest in response to UVB, Ras expression or actinomycin D (Table 2) [47, 167–169]. HPV E6 expression also allows cells to continue to proliferate in the presence of multinucleation as well as numerical and structural chromosomal abnormalities [123, 170].

HPV E6 inhibits apoptosis

While HPV E6 expression inhibits both the intrinsic and extrinsic apoptosis pathways, the intrinsic pathway is activated by damaged DNA and therefore is most relevant to this review. (For a more generalized discussion of the inhibition of the extrinsic apoptotic by HPV E6 see Yuan et al 2012 [171].) The best characterized means by which HPV E6 inhibits apoptosis is by degrading and otherwise inactivating p53 [172–175] (For a discussion of the role p53 in intrinsic apoptosis see Shen and White 2001 [176].) In addition to disrupting p53 activation, HPV E6 inhibits the intrinsic apoptotic pathway by directly altering the balance between pro- and anti-apoptotic members of the Bcl2 family (Table 2). Typically, cells respond to DNA damage shifting the balance between pro-apoptotic Bcl2 family members, like Bak and Bax, and anti-apoptotic Bcl2 family members, namely Bcl2, to favor apoptosis. To prevent Bcl2 family members from inducing apoptosis, HPV E6 decreases the levels of pro-apoptotic Bcl2 family members. Specifically, HPV E6 promotes

the degradation of Bak in a proteosome-dependent manner [177–181] and also reduces the level of Bax [182]. Furthermore, HPV E6 prevents the activation of other pro-apoptotic proteins, including the release of Apoptosis Inducing Factor (AIF) from the mitochondria [183] and the fragmentation of genomic DNA by DNA Fragmentation Factor 40 (DFF40) [184].

Additionally, HPV E6 also up regulates the expression of anti-apoptotic factors including Interleukin 6 and two members of the Inhibitors of Apoptosis (IAP) family, namely IAP-2 and Survivin (Table 2) [185–188]. HPV E6 further blocks apoptosis by impairing the activity of caspases that would otherwise execute apoptosis [189, 190]. For example, by interacting with the N-terminus of caspase 8, HPV E6 accelerates the degradation of caspases [191, 192].

Ultimately, many functional studies have emphasized the extent of HPV E6-mediated inhibition of apoptosis by inhibiting HPV E6's anti-apoptotic activities. This is particularly true for HPV transformed cells where inhibition of HPV E6 expression or disruption of HPV E6-mediated p53 degradation leads to apoptosis [193–195]. This is also the case when HPV E2 expression is reintroduced into HPV+ tumor cell lines. HPV E2 expression negatively regulates HPV E6 expression and is often lost in HPV+ tumors allowing increased HPV E6 expression. As a result, reestablishing HPV E2 expression in a HPV + tumor derived cell line represses HPV E6 expression, increases p53 levels and can induce p53-dependent and independent apoptosis [196, 197].

Concluding Statement

With regard to the DDR, the role of HPV proteins in the viral life cycle can be separated into two broad categories (Figure 1A, Table 1). The first set of viral proteins, HPV E1 and E2, activate a DNA damage response in order to replicate the viral genome. Upon activation, these viral proteins recruit DNA damage machinery to viral replication centers, likely taking advantage of repair proteins to elicit unlicensed replication. Indeed, multiple repair proteins are required for HPV genome replication. Importantly, cell cycle progression is also required for viral propagation and this represents a dilemma for the virus as cell cycle arrest often occurs concurrently with DDR.

The second set of viral proteins, namely HPV E6 and HPV E7, ensures that the cellular environment is conducive to viral replication. Specifically, HPV E7 deregulates cell cycle progression, by decoupling DNA damage repair and differentiation from cell cycle arrest, thus driving continual replication. Unbridled proliferation leads to significant genomic instability that, if not for HPV E6, would typically lead to cell cycle arrest or apoptosis. The primary function of HPV E6, with regard to viral replication, is to insure that the HPV E7-induced genomic instability as well as the activation of the DNA damage response by HPV E1, HPV E2 and HPV E7 does not elicit an apoptotic response. HPV E6 increases tolerance of both genomic instability and DDR by directly inactivating DDR proteins and by abrogating apoptotic signaling, most notably by promoting p53 degradation/inactivation. Thus, like many other viruses, HPV manipulates DDR to replicate the viral genome and to maintain a favorable cellular environment for viral propagation.

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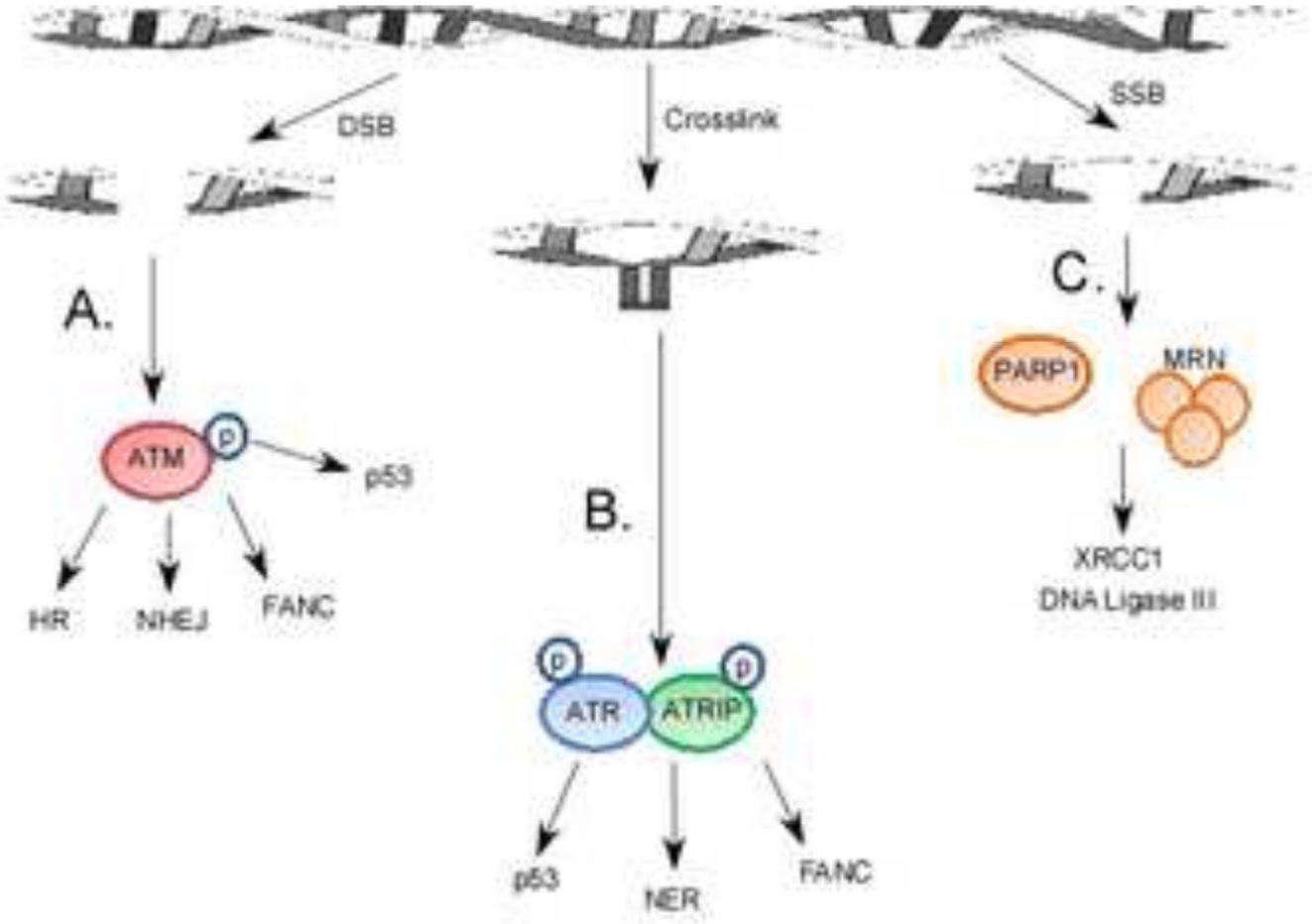

Figure 1.

Brief Overview of DNA Damage Repair: Three common types of DNA damage are depicted in this image as well as an summary of the pathways/proteins that are activated in response to each type of damage. A. A double strand break DNA break (DSB) most often results in the activation, by phosphorylation (indicated in this figure by a circled p), of the PI3 kinase ATM. Multiple pathways are downstream of ATM including homology dependent DSB repair (HR), non-homologous end joining (NHEJ), the Fanconi Anemia pathway (FANC), as well as the p53 signaling pathway. B. In response to intrastrand crosslinks (crosslink), ATR and its interacting partner ATRIP become activated and phosphorylated leading to the induction of several downstream pathways. The activated ATR/ATRIP complex induces the Fanconi Anemia repair and Nucleotide Excision Repair (NER) pathways, as well as the p53 signaling pathway. C. Finally, a single strand DNA break (SSB) causes the activation of both PARP1 and the MRN (MRE11, RAD50, NBS1) complex and ultimately together with XRCC1, DNA Ligase III as well as multiple other repair proteins fixes the lesion.

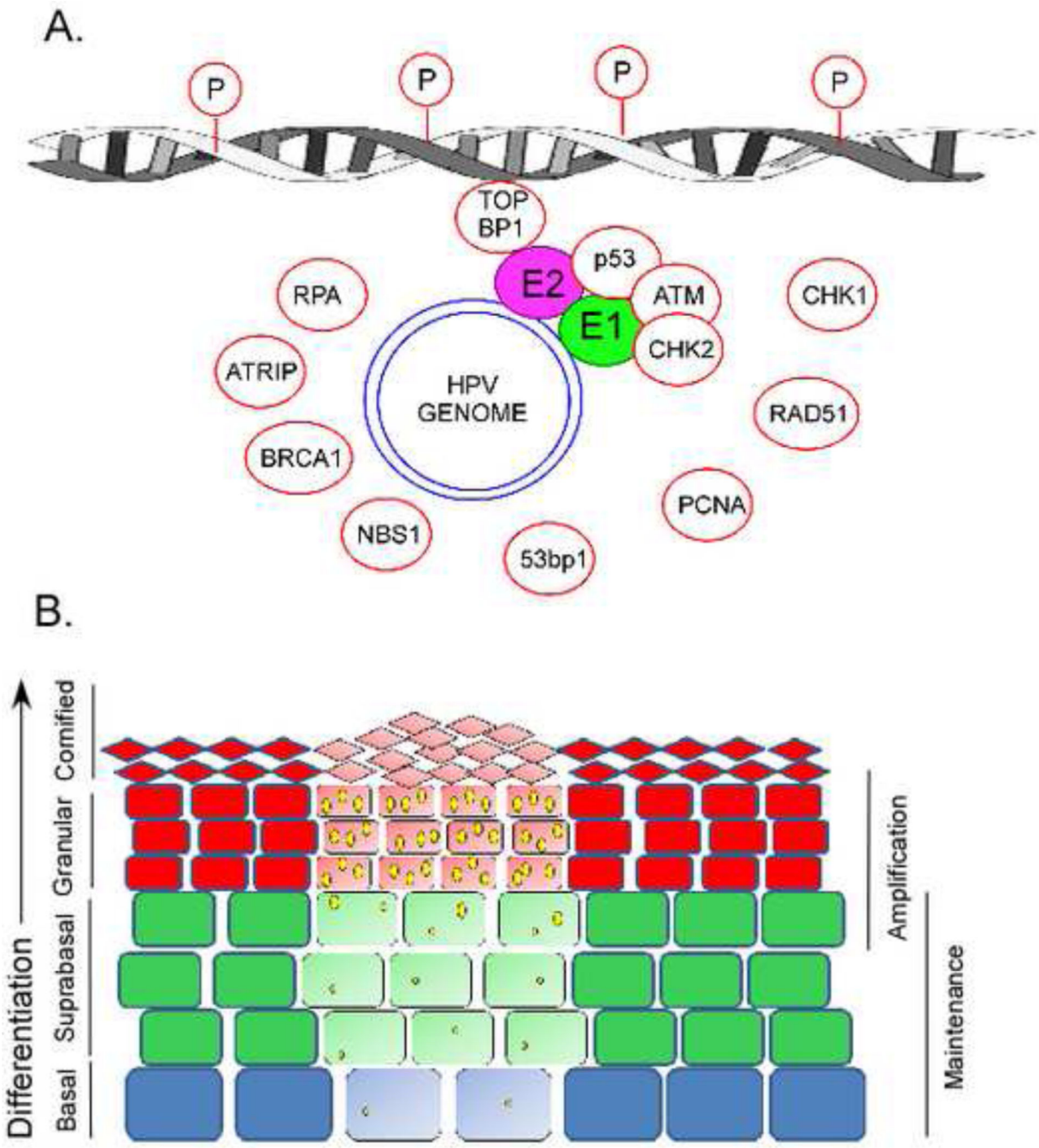

Figure 2.

HPV Replication and the Cellular DNA Damage Response: A. HPV E1 and E2 along with the HPV genome form viral replication centers. The replication center is depicted here along with the numerous DNA damage repair proteins that they are known to colocalize with. Several more direct interactions are also shown. Particular, HPV E2 tethers the viral genome to the cellular genome via an interaction with TOPBP1. Furthermore, HPV E1 and E2 colocalize with proteins involved in p53, ATR, and ATM signaling as well as enzymes involved in the homology dependent and independent repair of DSBs. Finally, viral

replication sites also colocalize to areas of phosphorylated H2AX, represented by a circled p. B. This figure shows a depiction of epithelium, HPV-infected cells are shown in lighter hues. Viral replication centers and the associated repair proteins depicted in Figure 2A are shown here as yellow dots. The magnitude of the induction of the cellular DNA damage response by HPV replication varies greatly between viral genome maintenance and viral genome amplification. During maintenance, the HPV genome is replicated is synchronized with cellular replication and viral copy number is relatively low. In contrast, as HPV-infected cells differentiate, the virus enters the amplification phase of its life cycle, when viral replication centers can greatly outnumber chromosomes. This increased HPV replication accompanying the transition from viral genome maintenance to amplification is depicted by both more numerous and larger yellow circles denoting both increase in quantity of viral replication centers as well as the enlarged DNA damage foci associated with this period of the viral lifecycle.

Table 1

Interactions among HPV proteins and host DNA damage repair pathways

	ATM Pathway			ATR Pathway		DSB Repair		
	Relevant Interactions	**Activity**	**Colocalization**	**Activity**	**Colocalization**	**Relevant Interactions**	**Activity**	**Colocalization**
E1		Activates Activates Activates	ATM Chk2 p53	Activates ATR			Activates	H2AX
E2		Activates Activates Activates	ATM Chk2 p53			TOPBP1	Activates	TOPBP1 H2AX
E6				Delayed ATR Activation		Homology Dependent Repair BARD1 BRCA1	Impairs Co-lps Interacts	
E7	ATM	Activates				BRCA2 BRCA1	Activates Inhibits	
HPV Replication Centers	ATM Chk2	Activates Activates	ATM Chk2	Activates CHK1	Chk1 ATRIP			BRCA1 RADS1 H2AX 53bp1 PCNA RPA NBS1

	p53 Signaling		SSB Repair		NER	
	Interactions	**Activity**	**Relevant Interactions**	**Activity**	**Activity**	**Relevant Proteins**
E2	p53 p300 p/CAF	Binds Binds Binds				
E6	p53 p300	Degrades and Inhibits Inhibits	MGMT XRCC1	Degrades Binds and Inhibits	Inhibits Delays Repair	Delays ATR Activation
E7	p300 p/CAF SRC1 p53	Inhibits Inhibits Inhibits Stabilizes				

	FA Pathway	
	Interactions	**Activity**
E6	BRCA1	Inhibits
E7	BRCA1 BRCA2 FANCD2	Inhibits Activates Activates

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Table 2

Interactions among HPV proteins and apoptotic/cell cycle regulatory proteins

	Apoptosis			**Cell Cycle Deregulation**	
	Pro-Apoptotic Proteins (Activated)	**Pro-Apoptotic Proteins (Inhibited)**	**Anti-Apoptotic Proteins (Activated)**	**Anti-Proliferative Proteins**	**Proliferative Proteins**
E1				Triggers S-phase Arrest	
E2	Activates Caspase 8				
E6	Activates Caspase 3, 7, 9	Degrades/Deactivates p53 Suppresses Caspase 8, 3, and 2 activation Inhibits Apoptosis Inducing Factor Degrades Bak Inhibits Bax	Increases BCl2, IAP2, and Survivin expression	Downregulates p21 Degrades/Deactivates p53 Inhibits pRB	Activates Cyclin A Activates CDK2
E7	Activates Caspase 3, 7, 9 Stabilizes p53 Induces “Trophic Sentinel” response			Degrades pRB Degrades p107 Degrades p130 Inactivates p21 Inhibits p27	Activates E2F1 Activates E2F2 Activates CDK2 Activates Cyclin A Activates Cyclin E Increases CDC25A expression