THE ACTIVATION AND CONSEQUENCES OF THE ATM MEDIATED DNA
DAMAGE RESPONSE IN HPV INFECTED CELLS

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ABSTRACT

Bryan Allen Johnson: The activation and consequences of the ATM mediated DNA damage response in HPV infected cells
(Under the direction of Cary Moody)

Infection with Human papillomavirus (HPV) is the most prevalent sexually transmitted disease in the world. From a public health standpoint, a subset of mucosa-tropic HPVs termed the high-risk genotypes are of most concern, as they are the causative agents of over 99 percent of cervical cancers and are increasingly linked to other forms of cancer. HPV initially infects the basal keratinocytes of the host epithelium and subsequently undergoes a life cycle tightly linked to the differentiation of its host cell. Despite having a small coding capacity, HPV is a master manipulator of the host cell, subverting a number pathways in order to ensure its own replication. Manipulation of the host cell is largely achieved through the expression of HPV's two major oncoproteins, E6 and E7, to dysregulate p53 and Rb-E2F signaling respectively.

This dissertation examines the function of the E7 protein in the viral life cycle. E7 expression has been linked previously to the activation of the ATM DNA damage repair pathway throughout infection and the induction of G_2 arrest in the upper layers of the stratified epithelium. Here I show that deletion of the Rb binding domain of E7 ablates its ability to increase the levels of ATM pathway proteins in HPV positive cells. Additionally, I demonstrate that E7 broadly upregulates the stability of DNA

repair factors to increase their levels, regulating the transcription of only a subset of

factors. I also show that the activity of the ATM kinase is necessary to increase the

levels of proteins regulating the G_2/M checkpoint, but does not play a role in

increasing the levels of most DNA repair factors. Together, these data establish a

model for ATM activation by E7 during HPV infection.

To my family, without your constant support I would have never have made it this far. And also to my friends, who reminded me to smile through the process. And finally, to the Spoetzl brewery and Shinerbock beer who filled in the gaps everything else missed.

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ATM   Ataxia telangiectasia mutated

ATP   Adenosine triphosphate

ATR   Ataxia telangiectasia and Rad3 related

BRCA1   Breast cancer 1

BIR   Break induced replication

Cdk   Cyclin dependent kinase

Cdk1   Cyclin dependent kinase 1

Cdk2   Cyclin dependent kinase 2

Chk1   Checkpoint kinase 1

Chk2   Checkpoint kinase 2

CKII   Casein kinase II

CR1   Conserved region 1

CR2   Conserved region 2

CR3   Conserved region 3

DSBR   Double stranded break repair

DDR   DNA damage response

DNA   Deoxyribonucleic acid

DNA-pk   DNA-dependent protein kinase

DSB   double stranded DNA break

E proteins   Early proteins

EGFR   Epidermal growth factor receptor

FoSTeS	fork stalling and template switching
G_1	Gap 1
G_2	Gap 2
HFK	Human foreskin keratinocyte
HPV	Human papillomavirus
HR	homologous recombination
HSPG	Heparin sulfate proteoglycan
Kb	Kilobases
L proteins	Late proteins
LCR	Long control region
MDC1	Mediator of DNA damage checkpoint 1
MH	microhomology
MMBIR	micrhomology-mediated break induced replication
MMEJ	microhomology-mediated end joining
MPF	Mitosis or Maturation promoting factor
MRN	MRE11-Rad50-NBS1
mRNA	messenger RNA
NBS1	Nijmegen breakage syndrome 1
NHEJ	non-homologous end joining
ORF	Open reading frame
PI3K	phosphoinositide 3-kinase
PIKK	PI3K related kinase
qPCR	Quantitative Real time polymerase chain reaction

Rb	Retinoblastoma
RNA	Ribonucleic acid
RPA	Replication protein A
SDSA	Synthesis-dependent strand annealing
SDS-page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SV40	Simian virus 40
URR	Upstream regulatory Region

CHAPTER 1: INTRODUCTION

OVERVIEW

Human papillomaviruses (HPVs) are small, non-enveloped, doubled stranded DNA viruses with a genome approximately 8 kilobases (Kb) in length. To date, there have been more than 100 genotypes of HPV identified, which can phylogenetically be divided into the alpha, beta, and gamma sub types. Infection with HPV is extremely prevalent and generally considered the most common sexually transmitted infection worldwide (48). From a public health standpoint, a subcategory of mucosatropic alpha papillomaviruses termed the "high-risk" genotypes (such as HPV 16, 18, 31, and 45) are of most concern, as they have been shown to be the etiological agent of over 99% of cervical cancers (167). Annually, high risk HPVs are responsible for some 490,000 cases of cervical cancer worldwide, resulting in 270,000 deaths (160). Increasingly, HPV infection has been linked to the development of other forms of cancer as well, including over 40% of oropharyngeal cancers (167). Given the disease burden caused by high risk HPVs, my dissertation research focused on the high-risk genotype HPV31. In particular, I focus on the role of the HPV31 E7 protein, a key manipulator of the host cell which ensures an environment conducive to viral replication.

INTRODUCTION

Organization of the viral genome. The prototypical Human papillomavirus (HPV) genome consists of a circular, double stranded DNA molecule approximately

8 kilobases (Kb) in length called an episome. As shown in Figure 1.1, eight viral

proteins are encoded in the genome consisting of the six early (E) proteins (E1, E2,

E4, E5, E6, and E7) and two late (L) structural proteins (L1 an L2). Each HPV

genome also contains a distinct non-coding region, referred to as the upstream

regulatory region (URR) or long control region (LCR). The URR (used hereafter) is

the primary site where viral replication and transcription is regulated, containing

HPV's origin of replication, numerous transcription factor binding sites, as well as

binding sites for the E1 and E2 proteins. High Risk HPVs contain two promoters,

termed the early and late promoters respectively, which are activated at different

phases in the viral life cycle. The early promoter is contained within the URR, while

the late promoter is positioned within the E7 open reading frame (ORF) (30). HPV

transcripts are heavily spliced to form multiple polycistronic RNAs that are translated

through leaky scanning by the ribosomal machinery (122).

Replication of the HPV genome is regulated through the viral E1 and E2

proteins, as evidenced by E1 an E2 binding sites located in close proximity to the

origin of replication (51, 153). The E1 protein is both an ATPase and a DNA

helicase, and recruits a variety of cellular replication proteins to the viral genome

(26, 72, 93, 98, 113). E1 itself binds only weakly to the viral origin, but its affinity is

greatly enhanced by E2 (51, 104, 130, 153). In addition to increasing E1's affinity for

the origin, E2 is thought to be the primary regulator of transcription through the early

promoter (29, 138). The functions of E4 and E5 are poorly understood, and are

mainly expressed during the productive phase of the life cycle where they are both

needed for productive replication. E4, also called E1^E4, consists of the first 5 amino

acids of E1 alternatively spliced with the E4 open reading frame. E4 is abundantly expressed in the upper layers of the stratified epithelium where it is hypothesized to play a role in virion release (39). E5 is thought to play a role in modulating EGFR signaling and in immune evasion (36). E6 and E7 are multifunctional and well-studied, and have been identified as the primary oncoproteins of high risk HPVs. Most famously, E6 abrogates p53 function by binding p53 and targeting it for proteasomal degradation in order to inhibit the induction of apoptosis and cell cycle arrest (70). Similarly, E7 binds and targets the pocket protein Rb for degradation, resulting in the activation of the E2F family of transcription factors to drive forward the cell cycle (124). The late proteins, L1 and L2, serve as the structural proteins of the HPV capsid and are only expressed late in infection (111).

THE HPV LIFE CYCLE

The life cycle of HPV can be divided into four distinct phases: viral entry, establishment, maintenance, and amplification. What follows is a brief discussion of each phase, including a description of important protein functions. What is lacking is a detailed discussion of E7 function, which will be discussed thoroughly in the following section. An overview of the HPV life cycle can be found in Figure 1.2.

Viral Entry and Establishment: HPV's entry into the cell is a multi-step, incompletely understood process. Infection by high risk HPV begins with a small wound in the mucosal epithelium called a microabrasion. A microabrasion is critical for HPV infection, as it gives HPV access to the basement membrane which is the site of initial binding in vivo (123). While bound to the basement membrane, L1 binds HPV's primary receptor, heparin sulfate proteoglycans (HSPGs), found on the
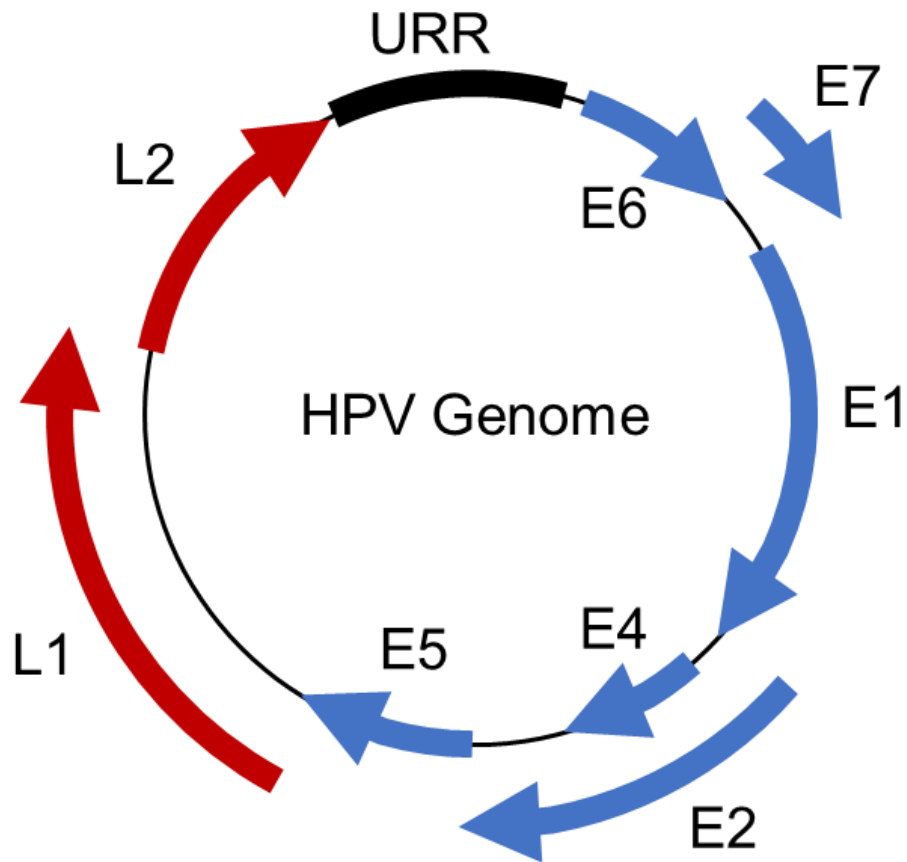
Figure 1.1 Map of the HPV genome: The viral open reading frames are indicated by blue arrows (early genes) and red arrows (late genes). The URR is indicated by the black box.

surface of keratinocytes in the lowermost or basal layer of the epithelium. Binding to HSPGs triggers a conformational change in the HPV capsid, exposing the L2 protein which then binds to an unknown co-receptor. Internalization then occurs by endocytosis (69). HPV then enters endosomes where it is eventually released into the cytoplasm and traffics to the nucleus (127). After nuclear entry, the HPV early promoter then becomes active, producing transcripts encoding for E1, E2, E6, and E7 (104, 153). HPV then replicates its genome to approximately 50-100 copies per

cell in a manner requiring the E1 and E2 proteins, before entering into the maintenance phase of the viral life cycle.

Maintenance Phase: After establishment replication, HPV replicates its genome synchronously with its host's DNA as it divides, maintaining viral copy number at 50-100 copies per cell. As mentioned above, E1 and E2 work together during this phase to regulate both the replication of the HPV genome and transcription through its early promoter by recruiting cellular factors to the genome (13, 100). Copy number is thought to be controlled in part by the E2 protein, which binds to 4 conserved sites flanking either side of the early promoter (142). E2 stimulates transcription from the early promoter at low concentrations while inhibiting transcription at high concentrations (32, 37, 145). Similarly, E2 binding to these sites has been shown to affect viral replication in a similar dose dependent manner (142). Thus, HPV self-regulates its copy number through an E2 controlled negative feedback loop, where increased levels of E2 inhibit additional viral replication by decreasing transcription of early genes (100). E2 also functions during maintenance phase to ensure proper distribution of viral genomes to the daughter cells as the host cell divides by tethering viral episomes to host chromosomes (101). E6 and E7 are also required for episomal maintenance (147). Collectively, E6 and E7 serve to create an environment conducive to replication through a wide variety of functions, including inhibition of apoptosis, subverting normal control of the cell cycle, and immune evasion (124, 154).

Amplification Phase: When an HPV infected basal keratinocyte divides, one daughter cell will form a new basal cell while another moves upward in epithelium

and begins to differentiate, marking entry into the productive phase of the viral life cycle. Differentiation activates HPV's late promoter, increasing the levels of a heterogeneous pool of mRNAs encoding E4, E5, L1, and L2, as well as the replication proteins E1 and E2 (57, 73, 146). Unlike the early promoter, expression from the late promoter occurs independent of E2 (141). The profile of transcriptional repressor and activators binding to known regulatory elements in the URR is known to be altered upon differentiation, suggesting that changes in transcription factors trigger late promoter activation (132). Expression from the early promoter is also maintained throughout the differentiation process, ensuring the continue expression of both E6 and E7 during differentiation (74). This is critical, as E7 works to create an environment permissive for productive replication by forcing infected keratinocytes to re-enter S-phase rather than exiting the cell cycle as uninfected keratinocytes (47, 53). This process is largely the result of E7's dysregulation of the Rb-E2F pathway through the degradation of the Rb, p107, and p130 pocket proteins, though a number of other E7 functions have been shown to be necessary for S-phase entry and productive replication (22, 35, 47, 52, 61, 66, 78, 102, 105, 165). It is then, after the host genome is finished replicating, that HPV productively replicates its genome through a process called amplification in cellular environment similar to G_2 arrest (10, 156). Increased levels of E1 and E2 resulting from activation of the late promoter drive genome amplification, increasing the number of HPV genomes per cell from 50-100 copies per cell to more than 1000 (83).

Both E4 and E5 are expressed primarily in the differentiating epithelia and are necessary for amplification, though their functions are poorly defined (36, 39). E4 is
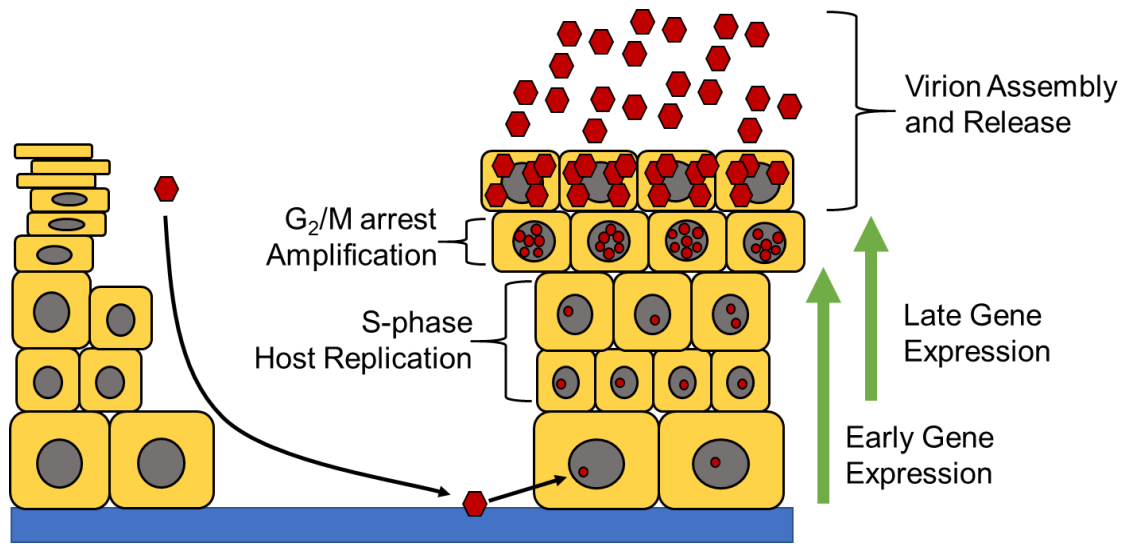
Figure 1.2 Overview of the HPV life cycle in the stratified epithelium. HPV particles
(indicated by red hexagons) infect basal keratinocytes after initially binding to basement
membrane. Upon infection, HPV will initially replicate its genome to approximately 50
copies per cell and begin early gene expression. From there, HPV undergoes
maintenance replication, replicating its genome synchronously with the cell as it divides.
Upon division, one daughter cell of the basal keratinocyte will move upwards in the
epithelium and begin the process of differentiation, triggering the productive phase of
the viral life cycle. Late gene expression will begin and host keratinocytes will re-enter
S-phase and replicate their genome. After S-phase and host replication is complete,
cells will arrest in G_2 and HPV will replicate its genome to over 1000 copies per cell
through a process termed amplification. Afterwards, infected keratinocytes will exit the
cell cycle, assemble virions, and progeny will be released from the cell.

expressed abundantly in the upper layers of the epithelium where viral amplification is occurring. E4 expression has been shown to re-locate cyclin B-Cdk1 complexes to the cytoplasm to prevent mitotic entry, suggesting that it may play a role in cell cycle regulation (31). Additionally, overexpression of E4 has shown to induce the collapse of the cytokeratin network, suggesting it may play a role in virion release (157). E5 function in the productive life cycle is unclear, though again modulation of EGFR and immune evasion have been suggested (39). The L1 and L2 structural proteins are also only highly expressed in the uppermost layers of the epithelium after amplification has been completed, where capsid assembly, encapsidation of the viral genome, and the release of mature virions is thought to occur (28).

THE ATM MEDIATED DNA DAMAGE RESPONSE AND G_2/M CHECKPOINT

The cellular response to DNA damage, collectively called "the DNA damage response" (DDR), is a robust and dynamic process involving a diverse set of biochemical pathways. Coordination of DDR is largely achieved through three class-IV phosphoinositide 3-kinase (PI3K)-related kinase (PIKK) kinases: ataxia telangiectasia mutated (ATM), ATM and Rad3 related (ATR), and DNA dependent protein kinase (DNA-PK), which phosphorylate hundreds of downstream targets to promote DNA repair, arrest the cell cycle, and prime the cell for apoptosis. Each of these kinases is activated in response to a distinct type of DNA damage and promotes a distinct mechanism of DNA repair, though cross talk between pathways has been firmly established (6). ATR activates the response to a number of types of DNA damage that result in single stranded DNA, such as stalled replication forks (25). DNA-PK and ATM on the other hand are both activated in response to double

stranded breaks in DNA (DSBs), but promote different DNA repair pathways. DNA-PK promotes DNA repair through the error prone non-homologous end joining (NHEJ) and is dominant when the cell is in G_1, while ATM promotes homologous repair (HR) which only occur in S-phase and G_2 when unbroken sister chromatids are available (71). Both ATR and ATM play a role in arresting the cell cycle in response to damage, giving the cell time to repair its DNA before cell division. This is accomplished in part through the phosphorylation of checkpoint kinase 1 and 2 (Chk1 and Chk2) by ATR and ATM. Phosphorylation activates Chk1 and Chk2 which in turn phosphorylate additional proteins to arrest the cell cycle (6).

Activation of both ATR and ATM have both been implicated in the productive replication of HPV, while the role of DNA-PK has not been examined. Levels of both ATR and ATM are increased in HPV infected cells, and inhibition of their kinase activity prevents genome amplification (2, 66, 105). Numerous targets of ATM including proteins involved in HR and cell cycle regulation have increased levels in HPV positive cells and are required for productive replication (3, 10, 20, 54, 105). My research has focused on the mechanism and consequences of ATM activation, thus it is necessary to examine in detail the ATM DDR pathway outside of infection in order to frame its role during HPV infection. We will only focus on the two most important functions of ATM for this dissertation: cell cycle arrest and HR.

Activation of the ATM pathway: As detailed in Figure 1.3, after a double stranded DNA break occurs the first step in activation of the ATM DDR is the recruitment of the MRN protein complex to the site of the break (6). The MRN
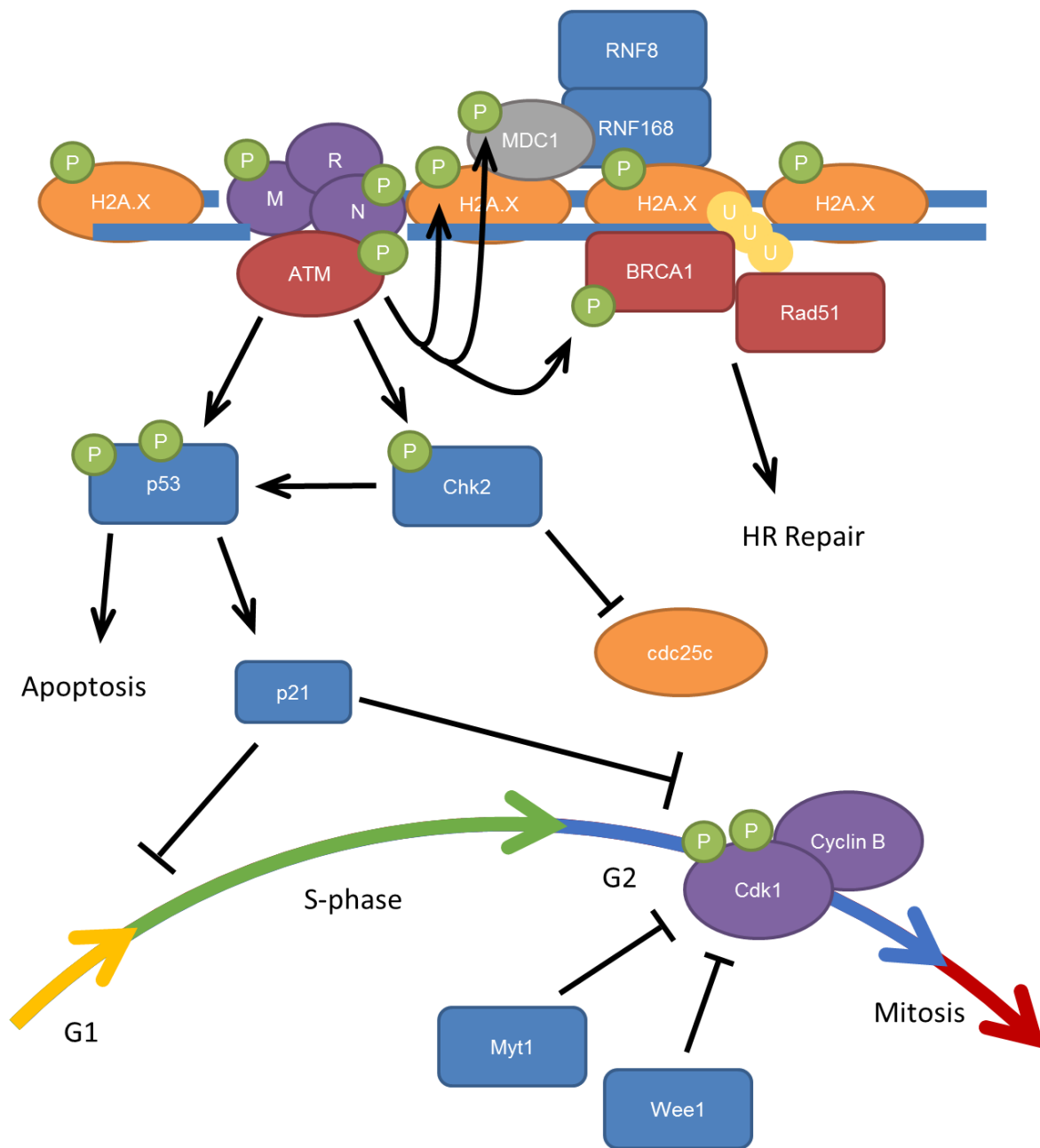
Figure 1.3 Diagram of ATM activation in response to DNA damage. Briefly, the MRN complex is recruited to the site of double stranded breaks in DNA. The MRN complex subsequently recruits and activates ATM. ATM then phosphorylates numerous downstream targets in order to facilitate DNA repair, cell cycle arrest, and promote apoptosis if repair is unsuccessful.

complex consists of three proteins: MRE11, Rad50, and NBS1. MRE11 possesses nuclease activity and is involved in DSB end resection to facilitate repair (114). Rad50 is an ATPase that binds to DNA, and is thought to aid in ATM activation by undergoing conformational changes (87). NBS1 interacts directly with ATM which is crucial for ATM's recruitment to DSBs (44, 163). Together, the MRN complex activates ATM by recruiting inactive ATM dimers to the site of the break, triggering ATM's auto-phosphorylation at Ser1981 and disassociation into active monomers (7, 88, 89). While the phosphorylation of ATM at Ser1981 is the canonical marker of ATM activation, additional post-translational modifications are also implicated in the full activation of the ATM pathway, including auto-phosphorylation at Ser367 and Ser1893 and the acetylation of Lys3016 by Tip60 (85, 143, 144). Upon activation, ATM then phosphorylates hundreds of downstream targets at a conserved ATR/ATM phosphorylation site consisting of either a Serine or Threonine followed by a Glutamine (S/TQ motif) (81).

      ATM mediated DNA repair by homologous recombination: A key function of the ATM pathway is to facilitate the repair of DNA by homologous recombination (HR). One target ATM uses to achieve this is the modified histone protein H2A.X which is phosphorylated on Ser139 (called γH2A.X when phosphorylated). γH2A.X acts as a marker of damaged DNA, recruiting additional repair factors to the site of the break. One such factor is MDC1 (also an ATM target), which co-operates with γH2A.X to recruit additional ATM molecules. These newly recruited ATM molecules then phosphorylated additional H2A.X proteins, creating a positive feedback loop that extends megabases from the site of the break along the damaged chromosome

(135). MDC1 binding also recruits RNF8/RNF168 which ubiquitylate H2A.X acting as a marker for the recruitment of BRCA1 and 53BP1 which promote HR and NHEJ, respectively (24, 94).

BRCA1 drives the decision toward HR by promotion of end resection, where nucleotides from one strand on each end of the DSB is removed in a 3'-5' manner in a manner dependent on MRE11's nuclease activity (19). The single stranded DNA produced by resection is then coated by a protein called RPA, and in the case of HR, subsequently by Rad51 by a process regulated by BRCA1 (159, 161). DNA repair then follows one of three sub pathways: double stranded break repair (DSBR), break induced replication (BIR), or synthesis-dependent strand annealing (SDSA) (71). The details of these pathways fall outside the scope of this dissertation, but are reviewed extensively elsewhere (24).

ATM induced cell cycle arrest: Distinct mechanisms exist that allow ATM to arrest the cell cycle at many different "check-points" in response to DSBs. When cells are in G_1, ATM activates p53 to prevent entry into S-phase through increased transcription of p21CIP1 (i.e. the G_1/S checkpoint). Activation of p53 is achieved both through direct phosphorylation by ATM as well as indirectly through phosphorylation by Chk2. Chk2 also phosphorylates cdc25a to inactivate cyclin dependent kinase 2 (Cdk2), whose activation is necessary for S-phase entry (58). Similarly, ATM has been shown to activate an intra-S checkpoint as well, though the details are poorly defined (137). Critically for the context of this dissertation, ATM activation prevents entry into mitosis from G_2 by inducing the G_2/M checkpoint. Transition from G_2 into mitosis is primarily regulated by cyclin dependent kinase 1

(Cdk1)-cyclin B kinase complexes, together known as the mitosis or maturation promoting factor (MPF) (120). In the absence of cell cycle arrest, cytoplasmic cyclin B levels increase during G_2 (119). To trigger entry into mitosis, cyclin B will bind Cdk1, activating Cdk1 kinase activity and both proteins will traffic to the nucleus (120). Mitotic entry also requires the activity of cdc25c, which removes inhibitory phosphorylations of Cdk1, Thr14 and Tyr15, which are conferred by the Myt1 and Wee1 kinases, respectively (38). During DNA damage, ATM inhibits this process by phosphorylating and activating checkpoint kinase 2 (Chk2) at Thr68 (1, 103). Chk2 then phosphorylates Serine 216 of the cdc25c phosphatase, inactivating it and leading to the accumulation of the inhibitory Thr14 and Tyr15 phosphorylations on Cdk1 (99, 115). Thus, ATM triggers G_2 arrest by initiating a phosphorylation cascade that eventually results in the inhibition of MPF, preventing mitotic entry.

FUNCTIONS OF THE E7 PROTEIN

E7 as a viral oncogene: Throughout the 1980s and early1990s a plethora of evidence emerged linking the E7 expression to cancer development. E6 and E7 were shown to be expressed in cell lines derived from cervical carcinomas (129, 162). Additionally, the integration of the HPV genome within the E2 ORF (commonly seen in HPV related cancers) increases proliferation through the increased expression of both E6 an E7 through the loss of E2 expression. (76). Silencing of E6 and E7 expression through the reintroduction of E2 triggers senescence of cancer cells in tissue culture, highlighting a role for E6 and E7 for in the growth of tumor cells (50, 56). Early studies demonstrated that E7 was the dominant of the two oncoproteins, as E7, but not E6, can alone transform immortalized and primary (in

13

co-operation with Ras) rodent cells (11, 12, 23, 43, 46, 77, 117, 155, 158). In the

same vein E7, but not E6, is sufficient to immortalize primary human epithelial cells,

though E6 can greatly enhance this function (60, 77, 106, 131). Mouse models

recapitulate these findings, where E7 expression has been shown to induce tumors

in various contexts (8, 9, 17, 75, 136, 149). Together, these studies clearly identify

E7 as a potent oncoprotein in HPV related cancers.

While a definitive model has yet to be established, E7 contributes to a number

of phenotypes that may play a role in its ability to promote oncogenesis. The classic

function of E7 is to bypass growth arrest through the dysregulation of the cell cycle

(33, 63, 109, 125, 128). Relatedly, and perhaps as a consequence of, E7 expression

is linked to a number of mitotic abnormalities including bypass of the mitotic spindle

checkpoint, multipolar mitoses, abnormal centrosome number, anaphase bridges,

and micronuclei formation (40-42, 96, 148). Expression of E7 is also linked to the

induction of replication and oxidative stress, DNA damage, activation of the DNA

damage response, loss of heterozygosity, and genomic instability, providing direct

mechanisms by which oncogenic mutations may occur (14, 21, 41, 105, 140). Taken

together, these studies demonstrate E7 likely promotes cancer development through multiple distinct mechanisms.
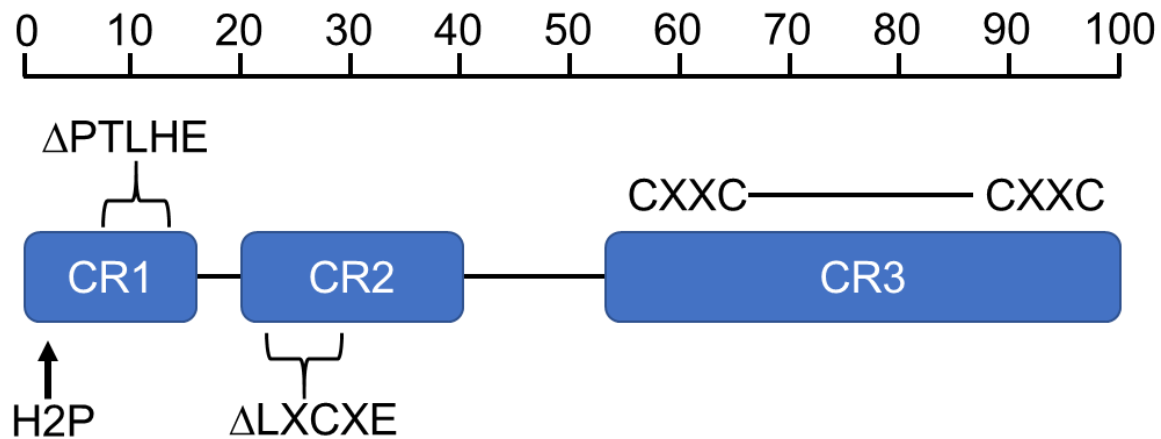
Figure 1.4 Diagram of the HPV E7 oncoprotein. Approximate amino acid position is indicated above the diagram. Blue boxes indicate major functional domains CR1, CR2, and CR3. The three most common mutants of E7 are indicated in their approximate positions in CR1 and CR2. A representation of the CXXC double zinc binding motif in CR2 is also indicated.

E7 Structure, posttranslational modifications, and localization: The HPV E7 protein varies in size from 98 to 105 amino acids depending on the genotype (82). Structural studies have demonstrated that E7 contains two distinct regions; a highly disordered N-terminal region and well-structured C-terminal zinc binding region (91, 110). As shown in Figure 1.4,The N-terminus of E7 contains two regions of conserved homology with other viruses, known as conserved region 1 and conserved region 2 (CR1 and CR2), separated by a small non-conserved linker (116, 117). Several phosphorylation sites have been identified in the N-terminus, including Threonines 5 and 7 (linked to the regulation of HPV 16 E7 stability) in CR1 as well as the casein kinase II recognition site (CKII) within CR2 (4, 12, 45, 90). Also

Phenotype	Citation
Protein Binding	
Loss of pRb/p107 binding	(33, 80, 92, 107, 116)
Loss of E2F/Cyclin A binding	(5)
Loss of IRF-1 binding	(112)
Loss of γ-tubulin binding	(108)
Loss of phosphorylated ATM binding	(105)
Loss of NBS1 binding	(3)
Other Relevant Phenotypes	
Fails to activate pRB-E2F dependent transcription	(18, 64, 116, 121)
Fails to destabilize pRb/p107/p130	(63, 80)
Reduces transformation of rodent cells	(18, 116)
Fails to bypass cell cycle checkpoints	(33, 63, 140)
Reduces p21 inhibition	(78)
Fails to increase cyclin E levels	(95)
Fails to Induce DNA synthesis	(27, 121)
Fails to block cyclin B degradation	(164)
Fails to immortalize normal human keratinocytes	(15, 63)
Fails to induce G_2 arrest	(10)

Table 1.1: Phenotypes associated with deletion of LXCXE motif. Abbreviated list of binding partners of E7 and phenotypes and associated with the loss the deletion of the Rb binding domain, with relevant citations.

within CR2 is the LXCXE motif or Rb binding domain that is responsible for binding E7's most famous binding partners, the cellular pocket proteins pRb, p107, and p130 (124). The C-terminus of E7, collectively referred to as conserved region 3 (CR3), consists of two CXXC zinc binding motifs separated by 29-30 amino acids. Within this region there is an S-phase specific phosphorylation site on Serine 71, the significance of which is poorly understood (97, 139). HPV E7 has been reported in both the nuclear and cytoplasmic fractions of host cells, and likewise contains both nuclear import and export sequences (84).

Anatomy of the Rb binding domain: The core Rb binding domain, also called the LXCXE motif, is a 5 amino acid motif within CR2. This motif is found in both adenovirus E1A and SV40 T-antigen, which lead to the identification as the site used by HPV E7 to bind to Rb (12, 117). Homology is highly conserved among HPV genotypes within this region. Three of the five amino acids, that is the leucine (L), cysteine (C), and glutamic acid (E), are conserved in a near universal manner across high and low risk HPV genotypes, while the other two amino acids (the two Xs in LXCXE) show some variation (124). In addition to this core sequence, the amino acid just prior to the LXCXE domain has been shown to be important in determining the affinity of HPV E7 for Rb. Among High Risk HPVs this residue is aspartic acid (D) and confers high affinity for Rb, while in low risk genotypes it is generally a glycine (G) and confers low Rb affinity. Swapping this amino acid alone among high and low risk HPV genotypes was shown to alter E7's affinity for Rb, decreasing affinity for high risk genotypes and increasing it for low risk (62).

Mutational analysis of E7 has long been used as an effective strategy for assaying E7 function, and the Rb binding domain is no exception. Perhaps the most common mutation used is the ΔLXCXE deletion, completely removing the motif from the protein. A number of different phenotypes have been reported for this mutant and are summarized in Table 1.1 (for a more extensive list see Tables 6 and 7 in 124). Most prominently, the ΔLXCXE mutant is unable to bind and destabilize pRb, p107, and p130 (63). E7-ΔLXCXE is unable to bind a diverse set of other cellular proteins as well, highlighting the critical nature of this motif. Biologically, this mutant ablates E7's to induce E2F dependent transcription, drive cells into S-phase, induce tumors in mice, or extend keratinocyte life span (27, 59, 63, 64, 116). In addition to the ΔLXCXE, two other less commonly used mutations of this site exist, C24G and C24S, both of which demonstrate similar though non-identical phenotypes from the deletion mutant (124). Together, these studies demonstrate the importance of the Rb binding domain in normal E7 function.

Dysregulation of the Cell Cycle by E7: The quintessential function of the E7 protein is the dysregulation of S-phase entry through the binding and degradation of the Rb protein. Outside the context of infection, the Rb protein serves to regulate entry into S-phase through the binding and inactivation of the E2F family of transcription factors. During the G_1/S transition, the Rb protein is hyperphosphorylated by Cdks, triggering the release of E2F transcription factors to drive the expression of S-phase genes. The E2F family consists of 8 proteins that serve as both activators and repressors of transcription. The activator E2Fs are E2F1-E2F3, while E2F4-E2F8 are the repressor E2Fs. Of these, E2F1-E2F3 are

regulated by Rb itself and are the members responsible for driving the expression of S-phase genes. E2F4 and E2F5 form complexes with the other two Rb family members, p107 and p130, and serve to repress the transcription of S-phase genes and regulate entry into G_1 from G_0. Binding to p107 and p130 is required for E2F4 and E2F5 function, and during G_1 p107 and p130 are also hyperphosphorylated by Cdks triggering the release of E2F4 and E2F5. E2F4 and E2F5 are then exported to the cytoplasm where they are unable to act as transcriptional repressors. Thus, entry into S-phase is largely controlled by the hyperphosphorylation of the Rb pocket protein family, which in turn increase S-phase gene expression through the inactivation of E2F4 and E2F5 and the activation of E2F1, E2F2, and E2F3. The functions of E2F6-E2F8 are poorly understood, though they are thought to function independently of pocket proteins in G_2 to shut off E2F-dependent transcription (150).

High risk HPV E7 subverts this carefully regulated process through direct interactions with all three members of the Rb pocket protein family. Classically, E7 binds to the pRb, p107, and p130 through its LXCXE motif within the CR2 region and marks it for proteasomal degradation (12, 16, 33, 34, 55, 63, 79, 80, 92, 166). Deletion of the LXCXE motif (ΔLHCYE) prevents binding and degradation of all three pocket proteins, as well as the immortalization of keratinocytes and transformation of rodent cells (15, 18, 63, 80, 116). Interestingly, sequences within the CR1 region of E7 are also required for the degradation of the Rb family members, but not for binding. Mutating E7's second residue from a Histidine to Proline (H2P) results in ablation of E7's ability to degrade, but not bind, Rb (33, 63). Experiments with the H2P mutant demonstrate that binding of E7 to Rb is alone not sufficient to drive

entry into S-phase, and that degradation of Rb is required. Rb degradation is also required for E7's ability to immortalize primary human keratinocytes, reiterating the importance of the E7-Rb-E2F relationship (63). Deletion of residues 6-10 (ΔPTLHE) of CR1 yielded similar phenotypes with regards to Rb degradation, E2F activation, and immortalization, confirming the importance of CR1 (33, 63, 80, 121). Finally, E7 is known to bind directly to both E2F1 and E2F6, demonstrating that E7 employs multiple mechanism to subvert Rb/E2F control of the cell cycle.

While the disruption of the Rb/E2F pathway is necessary for E7 to drive cells in to S-phase, it is not sufficient, insinuating that E7 must use other mechanisms to subvert the cell's control of S-phase entry (63). To this end, E7 has been shown to block the activity of the Cdk inhibitors p21 and p27 through residues in its C-terminal domain (52, 63, 78, 165). E7's ability to block p21 and p27 is necessary for E7's ability to induce S-phase entry, as evidenced by the introduction of E7 mutations that ablates E7's ability to do so (33, 63, 151). E7 also increases the activity of Cdk2 and interacts with cyclin E/Cdk2 and cyclin A/Cdk2 complexes through p107 (61, 102). Together, these data indicate that E7 uses multiple Rb independent mechanisms to promote S-phase entry. Within the context of the viral life cycle, this is most important in suprabasal keratinocytes, where E7 drives re-entry into S-phase upon the initiation of differentiation (22).

In addition to driving cells into S-phase, HPV infected cells have recently been shown to undergo a prolonged G_2 phase. Experiments with organotypic raft cultures demonstrate that in the stratified epithelium levels of HPV DNA increases after host DNA has finished replicating in cells with cytoplasmic cyclin B and low

levels of cyclin A, a marker of late G_2 (156). Later, in a seminal study by Banerjee et al., it was discovered that numerous signs of G_2 arrest were present in differentiating HPV infected keratinocytes (10). HPV infected cells grown in raft culture also exhibit high levels of cytoplasmic cdc25c and cytoplasmic Cdk1 in raft cultures, a phenotype consistent with G_2 arrest (38, 120). E7 expression was sufficient to induce cytoplasmic localization of cyclin B, Cdk1, and cdc25c, while E7-ΔLXCXE expression was not (10). This suggested that HPV induces these phenotypes through E7 in a manner dependent on its Rb binding domain. Western blot analysis of homogenized raft cultures demonstrated that total Cdk1 and cdc25c levels are elevated in HPV infected cells relative to uninfected cells. Cdc25c showed increased phosphorylation at Serine 216 while Cdk1 has increased phosphorylation at Thr14 and Tyr15, suggesting the G_2/M checkpoint is activated. Levels of Myt1 and Wee1, the kinases responsible for the inhibitory phosphorylation of Cdk1, were also increased. Again, expression of wild type E7 alone was sufficient to recapitulate these phenotypes (10). Together, these data suggest that E7 not only promotes entry into G_1/S to facilitate productive replication, but also induces the G_2/M checkpoint in order hold cells in an environment suitable for amplification.

Activation of the DNA damage Response: Manipulation of the DDR has been reported in numerous viruses, and HPV is no exception (65, 126). Recent research has revealed that activation of the DNA damage response plays a critical role in the HPV life cycle. In a landmark study, Moody and Laimins demonstrated that a number of DDR factors are increased in HPV31 positive cells across the differentiation dependent life cycle, including the ATM and Chk2 kinases. In that

study, it was demonstrated that pharmacological inhibition of the kinase activity of either ATM or Chk2 blocked genome amplification. Additionally, it was shown that expression of HPV31 E7 alone was sufficient to increase the phosphorylation and total levels of Chk2, suggesting that HPV E7 contributes to genome amplification in part through activation of the ATM pathway. Consistent with this, wild type HPV31 E7 was found to immuno-precipitate with ATM, while the HPV31 E7-ΔLHYCE mutant was not. Together, these data suggest that HPV activates the ATM pathway through E7 in order to facilitate the amplification of its genome (105)

Subsequent studies have further established a role for the ATM pathway in the replication of HPV. Several ATM pathway proteins localize to HPV replication foci, including ATM itself as well as prominent targets such as γH2A.X, Chk2, the MRN complex, Rad51, and BRCA1 (3, 54, 105). γH2A.X was shown to precipitate with HPV DNA, suggesting that it is present in the nucleosomes of HPV episomes (54, 105). Given γH2A.X's role in recruiting DNA repair factors to DSBs (24), this finding suggests a mechanism by which HPV may recruit DNA repair factors to its genome in order to facilitate replication. Outside of infection, the histone deacetylase SIRT1 is known to promote the recruitment of homologous repair proteins to DSBs in order to promote HR (152). During HPV infection, it was shown that SIRT1 binds HPV DNA where it regulates H1 and H4 acetylation. Pharmacological inhibition of SIRT1 blocked productive replication, while SIRT1 knockdown reduced levels NBS1 and Rad51 bound to the genome (86).Together, these data suggest that DDR proteins are actively recruited to the viral genome and that their recruitment may play a role in productive viral replication.

The levels of a number of DNA repair factors involved in activation of the ATM pathway and HR are increased in both HPV positive cells and cells expressing E7 alone (3, 20, 105). All three components of the MRN complex are increased dramatically in HPV31 positive cells and those stably expressing HPV31 E7. Like phosphorylated ATM, HPV 31 E7 was found to bind NBS1 and Rad50, but not MRE11. Disruption of the MRN complex through NBS1 knockdown was found to block amplification, as was pharmacological inhibition of MRE11 nuclease activity (3). Similarly, HPV31 E7 expression is sufficient to increase levels of HR proteins BRCA1 and Rad51. As was the case with NBS1, knockdown of either protein blocks genome amplification (20). Together, these studies demonstrate that proteins both upstream and downstream of ATM activation are necessary for productive HPV replication.

While the link between ATM activation and productive HPV replication is well established, the exact mechanism of ATM activation is less clear. While HPV E7 expression is sufficient to increase the levels of many proteins involved in the ATM-mediated DDR, the underlying mechanism E7 uses to activate the ATM is not known (3, 20, 105). Several studies have linked STAT5 activation to the activation of the ATM pathway. STAT5 is activated in HPV31 infected cells and 31 E7 expressing cells. Pharmacological inhibition of STAT5 suppressed ATM and Chk2 phosphorylation as well as amplification of the HPV31 genome, while knockdown of STAT5 decreased the levels ATM, Chk2, BRCA1, and Rad51 upon differentiation in calcium (68). STAT5 activation was shown to be required for activation of Tip60 in HPV31 positive cells, whose acetylation of ATM is necessary for ATM activation

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outside the context of infection (24, 67). However, while E7 expression can alone activate STAT5 and the ATM pathway it does not induce Tip60 activation, thus Tip60 activation alone cannot account for the E7 induced activation of the ATM pathway (67). Thus, the exact mechanism by which E7 activates STAT5 and its role in ATM activation has not been examined. Dysregulation of the Rb-E2F pathway is sufficient to induce DSBs, promote the formation of γH2A.X and MRE11 foci, and increase levels of ATM, Chk2, and Chk1 outside the context of infection (49, 118, 133, 134). Given that a key function of E7 is the binding and degradation of Rb, it is possible that Rb degradation also contributes to ATM activation in HPV positive cells. Thus, the exact mechanism of activation of the ATM pathway by E7 has not been established.

RATIONALE FOR DISSERTATION

Due to the relatively small size of the HPV genome, HPV must alter the cellular environment of its host cell in order to carry out its life cycle. The HPV E7 protein is one of the primary proteins responsible for achieving this goal. In a classic model, E7's primary function is to drive cells into S-phase in order promote the expression of host replication factors needed for replication of the HPV genome. Recent studies have begun to expand this view. The link between HPV E7's activation of DDR and HPV genome amplification is a function of E7 that at face value is largely unrelated to S-phase entry, but the mechanistic details of how E7 induces ATM activation are incomplete. Additionally, the observation that E7 expression alone is sufficient to induce the G_2/M checkpoint suggests that E7

manipulates the cell cycle in a more complex manner than merely driving suprabasal cells into S-phase.

In Chapter 2, I describe how the activation of the ATM pathway in HPV31 positive and E7 expressing cells requires the Rb binding domain. Specifically, I demonstrate that the Rb binding domain is necessary for increased protein levels of ATM and its target Chk2, the ATR target Chk1, all three MRN complex proteins, as well as the HR proteins BRCA1 and Rad51. This phenotype was conserved in both HPV31 positive cells and cells expressing 31 E7 alone. Additionally, I show that increases in DDR factors do not only occur at the level of increased mRNA transcription, as might be expected if triggered by the dysregulation of the Rb-E2F pathway. Instead, HPV31 E7 greatly increases the half-lives of these proteins in a manner dependent on the Rb binding domain.

In Chapter 3, I explore preliminary data with regards to ATM's role in inducing the G_2/M checkpoint. I demonstrate that while inhibition of ATM kinase activity has no effect on the levels of DDR factors, upon differentiation it does cause a decrease in the levels of proteins regulating G_2 arrest. Together, these data suggest an additional role for E7 and the ATM pathway in HPV's differentiation dependent life cycle.

As a whole, my research continues the elucidation of a detailed life cycle of HPV. Understanding the HPV life cycle will facilitate the development of novel therapeutics to combat the replication and spread of HPV. Understanding the function of E7 within the HPV life cycle may also illuminate mechanisms by which E7 promotes oncogenesis.

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CHAPTER 2: THE RB BINDING DOMAIN OF HPV31 E7 IS REQUIRED TO MAINTAIN HIGH LEVELS OF DNA REPAIR FACTORS IN INFECTED CELLS[1]

OVERVIEW

Human papillomaviruses (HPV) exhibit constitutive activation of ATM and ATR DNA damage response (DDR) pathways, which are required for productive viral replication. Expression of HPV31 E7 alone is sufficient to activate the DDR through an unknown mechanism. Here, we demonstrate that the E7 Rb binding domain is required to increase levels of many DDR proteins, including ATM, Chk2, Chk1, the MRN components MRE11, Rad50, and NBS1, as well as the homologous recombination repair proteins BRCA1 and Rad51. Interestingly, we have found that the increase in these DNA repair proteins does not occur solely at the level of transcription, but that E7 broadly increases the half-life of these DDR factors, a phenotype that is lost in the E7 Rb binding mutant. These data suggest that HPV-31 upregulates DNA repair factors necessary for replication by increasing protein half-life in a manner requiring the E7 Rb binding domain.

INTRODUCTION

High-risk human papillomaviruses (HPV) are the etiological agents of cervical cancer, and are also associated with other genital malignancies, as well as an increasing number of head and neck cancers (47). HPV has adapted its life cycle to be linked closely with epithelial differentiation, with late viral events being restricted to the uppermost layers of the epithelium (27). HPV is thought to infect dividing, basal cells of the stratified epithelium through a microwound, where upon entry into the nucleus, the virus is maintained as a low copy episome. As an infected cell divides, one daughter cell migrates upward and initiates differentiation. Differentiation triggers the productive phase of the viral life cycle, which requires cellular factors. HPV maintains differentiating cells active in the cell cycle through viral gene expression, allowing for initiation of DNA synthesis and amplification of viral genomes to thousands of copies per cell. Late gene expression, as well as virion assembly and release occur concomitantly with productive replication (30).

Previous studies demonstrated that the productive replication of high-risk HPV31 requires activation of an ATM-dependent DNA damage response (DDR) (31). ATM is a serine/threonine kinase that is activated primarily in response to double strand DNA breaks (DSBs), resulting in phosphorylation of a variety of substrates important in activating cell cycle checkpoints, as well as DNA repair (8). If left unrepaired, DSBs have the potential to generate chromosomal translocations, aneuploidy, and increased incidence of malignancy (1, 14). Though the DNA damage response plays a crucial role in the maintenance of genomic stability, many viruses have been shown to exploit repair pathways to facilitate replication (15, 39). We have previously shown that DNA repair

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factors localize to sites of HPV replication, indicating a direct role for these factors in efficient viral replication (13). In support of this, we have found that the histone variant H2AX, one of the first targets of ATM, is bound to HPV DNA (13). In addition, we recently demonstrated that the MRN complex (Mre11, Rad50, Nbs1), which facilitates ATM activation in response to ionizing radiation (24, 25, 35), is localized to HPV31 genomes and required for productive replication (2). In addition, we have found that Rad51 and BRCA1, two factors essential for repair of DSBs through homologous recombination (8), are required for productive viral replication (7). Although numerous studies support a role for the ATM pathway in productive replication of HPV (2, 7, 13, 17, 18, 31), the mechanism by which ATM is activated in HPV-infected cells remains unclear.

The E6 and E7 oncoproteins of high-risk HPV types contribute to carcinogenesis largely through their ability to target the tumor suppressors p53 and Rb for degradation, respectively (19, 38). This is especially important upon differentiation, as E6 and E7 ensure virus production by promoting S-phase re-entry of a subset of differentiating cells (30). The ability of E6 and E7 to target critical regulators of cell cycle progression results in the bypass of checkpoints normally involved in the elimination of abnormal cells (32). While this is necessary for viral replication, checkpoint abrogation can also result in genomic instability in HPV-immortalized cells that eventually leads to cancer (42). High-risk E7 expression has been shown to lead to DSB induction and genomic instability in a manner thought to be dependent on Rb inactivation and deregulation of E2F transcription factors (5, 10, 36). Inactivation of Rb outside the context of HPV infection is sufficient to induce DSBs, formation of □H2AX and MRE11 positive foci, and

increase levels of ATM, Chk2, and Chk1 (12, 36, 40, 41). Previous studies demonstrated that expression of high-risk HPV31 E7 alone is sufficient to induce ATM signaling (31), raising the possibility that E7 contributes to the differentiation-dependent phase of the life cycle through modulation of the DDR. More recent studies by Hong et al. demonstrated that the phosphorylation of STAT5 is required for ATM activation in HPV31 positive cells, potentially through E7-mediated Rb inactivation (17, 18). Studies by this same group have also provided a link between STAT5, activation of the ATR DNA damage kinase and productive replication of HPV31 (16). Although Rb inactivation by E7 is important for providing an environment conducive to productive viral replication, whether a direct link exists between Rb binding and ATM/ATR activation has not been demonstrated. However, recent studies linked the development of female reproductive tract cancers, as well as head and neck cancers in HPV16 transgenic mice to E7-mediated inactivation of pocket proteins (Rb, p107, p130) and resultant DNA damage (33, 34).

In this study, we investigated whether the Rb binding domain of E7 is required for activation of ATM, as well as ATR signaling pathways in HPV31 positive cells. We have found that deletion of the Rb binding domain in the context of the HPV31 genome results in decreased levels of DNA repair factors compared to cells maintaining wild-type HPV31 genomes. A similar phenotype was observed in cells expressing wild-type HPV31 E7 alone compared to cells expressing an E7 Rb binding mutant. Interestingly, we found that E7 maintains high levels of DNA repair factors required for productive replication through increased protein stability, rather than exclusively through increased gene expression, with exceptions limited to the ATR target Chk1, and the homologous

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recombination repair factors BRCA1 and Rad51. Together, these data suggests that the E7 Rb binding domain is important for increased ATM/ATR activation by virtue of increasing total protein levels through increased protein stability, and that increases in DNA repair factor levels in HPV positive cells depends largely on the ability of E7 to bind and target Rb for degradation.

MATERIALS AND METHODS

Cell Culture: Human foreskin keratinocytes (HFKs) were isolated from neonatal foreskins as previously described and were cultured in E medium supplemented with 5 ng/ml mouse epidermal growth factor (EGF; BD Biosciences) (43). HFK-31, HFK-31 ΔLHCYE, pLXSN-31 E7, pLXSN-31 E7 ΔLHCYE cells were also cultured in E medium supplemented with 5 ng/ml mouse epidermal growth factor. All lines were cultured in the presence of mitomycin C-treated J2 3T3 fibroblast feeder cells, as previously described (43). J2 feeder cells were removed from HPV-positive cells with 1mM EDTA in phospho-buffered saline (PBS) as necessary.

Plasmids: The pBR322-HPV31 plasmids containing the wild-type HPV31, HPV31 E7 ΔLHCYE mutant genomes have been previously described (20, 26). Briefly, the E7 ΔLHCYE plasmid contains an in-frame deletion of the Rb binding site. The pLXSN retroviral vectors encoding wild-type HPV31 E7 and the E7-Rb binding mutant (ΔLHCYE) have been described previously (26).

Generation of HFK-31 lines: HFKs maintaining wild type HPV31, as well as mutant HPV 31 genomes (HFK-31 E7 ΔLHCYE) (43). Briefly, HPV genomes were digested with HindIII to release them from the pBR322 plasmid backbone. T4 DNA ligase (Life Technologies) was then used to re-ligate the excised HPV genomes. HFKs

were co-transfected with 2.5μg of ligated HPV genomes and 2.5μg of pSV2-neo using PolyJet transfection reagent as per the manufacturer's instructions (Signagen Laboratories), followed by selection in G418 (Sigma). Surviving populations were expanded for further analysis.

Keratinocyte Differentiation: Differentiation of keratinocytes was performed by suspending cells in 1.5% methylcellulose, as previously described (43). Cells were harvested as an undifferentiated sample (T0), as well as 24 and 48hrs after suspension in methylcellulose. At each time point, DNA, RNA and protein were harvested.

Western Blot Analysis: Whole cell lysates were harvested by lysing cell pellets in RIPA buffer supplemented with Complete Mini protease inhibitor (Thermo Scientific) and PhoSTOP phosphatase inhibitor tablets (Roche). Total protein concentrations were determined by Bio-Rad protein assay (Bio-Rad). Equal amounts of protein were separated by SDS-page and transferred to polyvinylidene difluoride (PVDF) membranes. (Immobilon-P; Millipore). The following primary antibodies were used: phospho-ATM Ser1981, Chk1, NBS1 (Abcam); ATM (Bethyl laboratories); phospho-Chk1 Ser345, phospho-Chk2 Ser68, Chk2, E2F1 (Cell Signaling Technology); E2F2, E2F3, GAPDH, Rad51 (Santa Cruz); MRE11, Rad50, and BRCA1 (GeneTex). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit (Cell Signaling Technology) and HRP-conjugated anti-mouse (GE Life Sciences). Western blots were developed using Enhanced Chemiluminescence Prime blotting substrate (GE Life Sciences). Images were captured with the Biorad ChemidocMP imaging system, and analyzed with Biorad Imagelab 5.0 software.

Southern Blot Analysis: DNA isolation and Southern blotting were performed as previously described (11). Briefly, cells were harvested in DNA lysis buffer (400mM NaCl, 10mM Tis pH 7.5 and 10mM EDTA), then lysed by the addition of 30uL 20% SDS. Samples were subsequently treated with 15ul of 10mg/mL proteinase K overnight at 37˚C. DNA was extracted using phenol chloroform, followed by ethanol precipitation in the presence of sodium acetate. 5ug of DNA was digested with either BamHI (New England Biolabs) (does not cut the genome), or HindIII (New England Biolabs) (cuts the genome once). DNAs were resolved on a 0.8% agarose gel for 15 h at 40 V and were then transferred to a positively charged nylon membrane (Immobilon-Ny+; EMD Millipore). The DNA was fixed to the membrane via UV irradiation and then hybridized to a radioactive DNA probe consisting of ^{32}P-labeled linearized HPV31 genome.

Real-time PCR: RNA was extracted from primary HFKs, HFK-31, HFK-31 ΔLHCYE, pLXSN, pLXSN-31 E7, and pLXSN-31 E7 ΔLHCYE cells using RNA STAT 60 (Tel-test). DNA was removed from samples via treatment with RQ1 DNAse (Promega) via the manufacturer's protocol. cDNA was made using the iScript reverse transcription kit (Bio-Rad). Quantitative RT-PCR was performed in triplicate on 50 ng of cDNA using 375 nM primers and iTaq Universal SYBR Green Supermix (Bio-Rad) in a total reaction volume of 10 μl. Reactions were performed using an ABI QuantStudio 6 Flex thermal cycler and analyzed with version 1.0 of the QuantStudio 6 and 7 Flex software. The thermal profile used for PCR is as follows: 10 min denaturation at 95ºC followed by 40 cycles of 95ºC for 15 sec, then 60 sec at 60ºC (ATM, Chk2, Chk1, MRE11, Rad50, NBS1, E2F2, E7) or 63ºC (BRCA1, Rad51, E2F1), followed by 72ºC for 30 sec. A melt curve was run to ensure primer annealing. Relative transcript levels were determined

using the threshold cycle method (ΔΔCT) with GAPDH serving as an endogenous

control gene. Values were normalized relative to transcript levels of primary HFKs or

pLXSN control cells. The primer sequences are as follows: ATM Forward, 5′-

TGTTCCAGGACACGAAGGGAGA-3′; ATM Reverse, 5′-

CAGGGTTCTCAGCACTATGGGA-3′; BRCA1 Forward, 5′-

CTGAAGACTGCTCAGGGCTATC-3′; BRCA1 Reverse, 5′-

AGGGTAGCTGTTAGAAGGCTGG-3′; Chk1 Forward 5′-

TGAGAATCCATCAGCAAGAATTACC-3′; Chk1 Reverse, 5′-

ATCCACTGGGAGACTCTGACACA-3′; Chk2 Forward, 5′-

GCAGCAGTGCCTGTTCACA-3′; Chk2 Reverse, 5′-TGGATATGCCCTGGGACTGT-3′;

E2F1 Reverse 5′-ATCTGTGGTGAGGGATGAGG-3′ E2F2 Forward, 5′-

CTCTCTGAGCTTCAAGCACCTG-3′; E2F2 Reverse, 5′-

CTTGACGGCAATCACTGTCTGC-3′; HPV31 E7 Forward, 5′-

ACACCTACGTTGCAAGACTATG-3′; GAPDH Forward, 5′-

CTGTTGCTGTAGCCAAATTCGT-3′; GAPDH Reverse, 5′-

ACCCACTCCTCCACCTTTGAC-3′; HPV31 E7 Reverse, 5′-

CGAATATCTACTTGTGTGCTCTGT-3′, Mre11 Forward, 5′-

GCCTTCCCGAAATGTCACTA-3′; Mre11 Reverse, 5′-TTCAAAATCAACCCCTTTCG-3′;

NBS1 Forward, 5′-TCTGTCAGGACGGCAGGAAAGA-3′; NBS1 Reverse, 5′-

CACCCCAAAGACAACTGCGGA-3′; Rad50 Forward, 5′-

GGAAGAGCAGTTGTCCAGTTACG-3′; Rad50 Reverse, 5′-

GAGTAAACTGCTGTGGCTCCAG-3′; Rad51 Forward, 5′-

TCTCTGGCAGTGATGTCCTGGA-3′, Rad51 Reverse, 5′-

TAAAGGGCGGTGGCACTGTCTA-3′.

Measurement of Protein Half-Life: Primary HFKs, HFK-31, HFK-31 ΔLHCYE,

pLXSN, pLXSN-31E7 and pLXSN-31E7 ΔLHCYE cells were grown in 10 cm dishes until

~80% confluency. Whole cell lysates were then harvested from one dish for the 0 hr

time point and at the indicated time points after treatment with 50 μg/ml cycloheximide.

J2 fibroblasts were removed prior to harvest using Versene (1mM EDTA in PBS).

Western blot analysis was performed using 50 μg of total protein as described above.

Westerns were digitally imaged using the Bio-Rad Chemidoc MP system, and

densitometry was performed with the Biorad ImageLab 5.0 software.
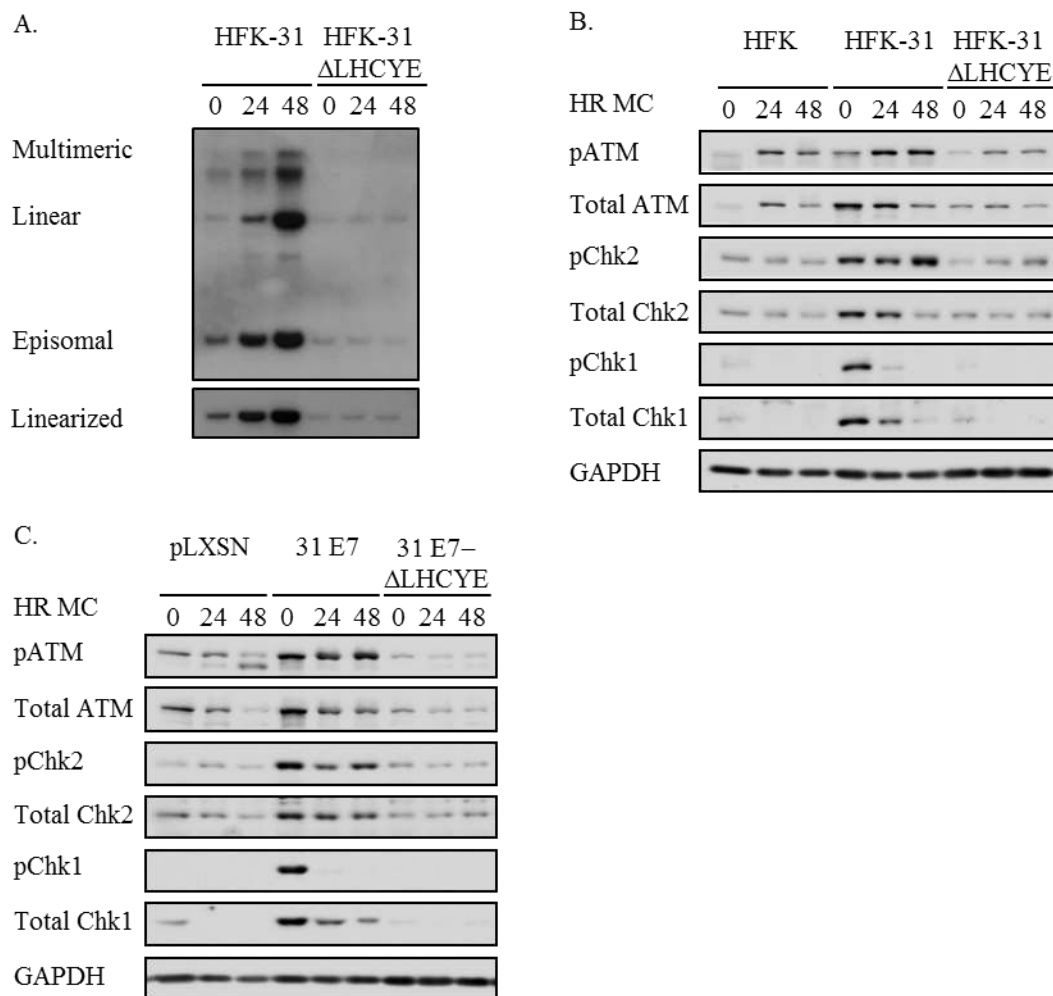
RESULTS

The Rb binding domain of E7 is required for ATM and ATR activation in

HPV31 positive cells: To examine the importance of the E7 Rb binding domain to DDR

activation and maintenance of high levels of DDR factors in HPV31 positive cells, we

generated human foreskin keratinocyte (HFKs) lines that maintain either wild-type

HPV31 genomes (HFK-31), or genomes containing a mutation in the E7 LXCXE Rb

binding domain (HFK-31 ΔLHCYE). Previous studies demonstrated that this mutation

does not alter the stability of E7, and that HFK-31 ΔLHCYE mutant genomes are

maintained extrachromosomally, though copy number decreases over time compared to

wild-type genomes (26). In addition, these studies showed that the E7 Rb binding site is

required for efficient amplification of viral genomes upon differentiation (26), and we

have found similar results in this study (Figure 2.1A). To determine the effect of the

ΔLHCYE mutation on DDR activation throughout the viral life cycle, cells were

harvested both prior to and after the induction of epithelial differentiation in methylcellulose, a method commonly used to activate the productive phase of the viral life cycle. We first examined the importance of the E7 Rb binding domain on the activation of the ATM and ATR pathways. As shown in Figure 2.1B, both the phosphorylated and total levels of ATM, the ATM target Chk2, and the ATR target Chk1 were increased in HFK-31 cells compared to uninfected HFKs, indicating activation of ATM/ATR signaling pathways, as published previously (16, 31). While the levels of total ATM and Chk2 decreased in HFK-31 positive cells upon differentiation, the phosphorylated levels of ATM and Chk2 remained elevated, indicating DDR activation is increased during the productive phase of the viral life cycle. In contrast, both phosphorylated and total levels of Chk1 decreased upon differentiation, despite being required for productive replication (16). Interestingly, we found that the levels of phosphorylated and total ATM and Chk2, as well as Chk1 dramatically decreased in the HFK-31 ΔLHCYE cells at all time points, exhibiting levels similar to that found in the uninfected HFKs (Figure 2.1B). These data suggest that in the setting of an HPV infection, the Rb binding domain of E7 is necessary for ATM and ATR activation.

Previous studies demonstrated that HPV31 E7 expression is alone sufficient to activate the ATM and ATR pathways in keratinocytes (16, 31). Additionally, studies by other groups have shown that E7 expression alone induces DNA damage and activates the DDR (10, 36). To determine if the E7 Rb binding domain is required for DDR activation outside the context of viral infection, we stably transduced HFKs with either a retroviral control vector (pLXSN), a vector encoding wild type HPV31 E7, or HPV31 E7 containing the ΔLHCYE mutation. We then analyzed ATM and ATR activation from both

undifferentiated and methylcellulose-differentiated cells by Western blot analysis. As shown in Figure 2.1C, HFKs expressing wild-type HPV31 E7 exhibited increased levels of both phosphorylated and total ATM, Chk2, and Chk1 relative to control cells, confirming that E7 expression alone is sufficient to activate ATM and ATR signaling pathways. However, this phenotype was lost in cells expressing the E7 ΔLHCYE mutant (Figure 2.1C). Overall, these results indicate that maintenance of high levels of ATM, Chk2 and Chk1 in HPV31 positive cells requires the Rb binding domain of E7, which likely contributes to the ability of HPV to activate ATM and ATR signaling pathways.

**Figure 2.1. The Rb binding domain of E7 is required for ATM and ATR activation in HPV31 positive and E7-expressing keratinocytes.** (A) Southern blot analysis performed on DNA harvested at the indicated time points from human foreskin keratinocytes maintaining wild-type HPV31 genomes (HFK-31), as well as HFK-31 cells containing a mutation in the E7 Rb binding site (HFK-31 ΔLHCYE). DNA was digested with either BamH1, which does not cut the viral genome, or HindIII, which cuts the genome once. The HPV31 genome was used as a probe. (B) Western blot analysis was performed on lysates harvested from HFKs, HFK-31 cells, or HFK-31 ΔLHCYE cells. Lysates were harvested from undifferentiated cells (T0), as well as after 24 and 48hr differentiation in methylcellulose (MC). Primary antibodies used were phosphorylated ATM on Ser1981 (pATM), total ATM, phosphorylated Chk2 on Ser68 (pChk2), total Chk2, phosphorylated Chk1 on Ser345 (pChk1), and total Chk1. GAPDH served as a loading control. Shown are blots representative of five independent experiments across four HFK backgrounds. (B) Whole cell lysates were harvested from undifferentiated (T0) as well as differentiated (24, 48hr MC) HFKs stably transduced with either a retroviral control construct (pLXSN) or retroviral constructs expressing wild-type HPV31 E7 (pLXSN-31 E7), or HPV31 E7 containing a mutation in the Rb binding domain (pLXSN-31 E7 ΔLHCYE). Immunoblotting was performed as described in panel A. Western blots shown are representative of three independent experiments across two HFK backgrounds.

Maintenance of high levels of DNA repair factors in HPV31 positive cells requires the Rb binding domain of E7: The MRN protein complex (consisting of MRE11, Rad50, and NBS1) is a DNA damage sensor that activates the DDR by

recognizing DSBs and activating ATM (24, 25, 35). Previously, we showed that the levels of MRN components are increased in HPV31 positive cells, and that the maintenance of the MRN complex is necessary for productive viral replication (2). We next wanted to determine if the E7 Rb binding domain is also required for the maintenance of high levels of MRN complex members in HPV31 positive cells. As shown in Figure 2.2A, levels of all three MRN components were elevated in undifferentiated HFK-31 cells compared to primary HFKs, and this phenotype was maintained upon differentiation in methylcellulose. However, this phenotype was lost in cells containing E7 ΔLHCYE mutant genomes, with levels of MRN components resembling those found in HFKs (Figure 2.2A). Similar results were observed in E7-expressing lines, with cells expressing the E7 ΔLHCYE mutant exhibiting levels of MRN components similar to that of HFKs (Figure 2.2B). Together, these data suggest that the Rb binding site of E7 is necessary for maintenance of high levels of MRN components in HPV31 positive cells.

BRCA1 and Rad51 are two proteins essential to homologous recombination, a DSB repair mechanism that also requires ATM activity (8). Previously, we demonstrated that levels of BRCA1 and Rad51 are increased in HPV31 positive cells and are required for productive viral replication (7). As shown in Figure 2.2C, HFK-31 cells exhibited increased levels of BRCA1 and Rad51 compared to HFKs, as previously reported (7, 13). However, the E7 ΔLHCYE mutation resulted in decreased BRCA1 and Rad51 levels, similar to those found in HFKs, indicating the E7 Rb binding domain is required for increased levels of BRCA1 and Rad51 in HPV31 positive cells. These findings were recapitulated in cells expressing E7 alone, with the E7 Rb binding domain being

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required for maintenance of high levels of BRCA1 and Rad51 (Figure 2.2D). Overall,

these results indicate that E7 expression is necessary for HPV31 to increase the levels

of DDR factors and that this increase requires the E7 Rb binding domain.
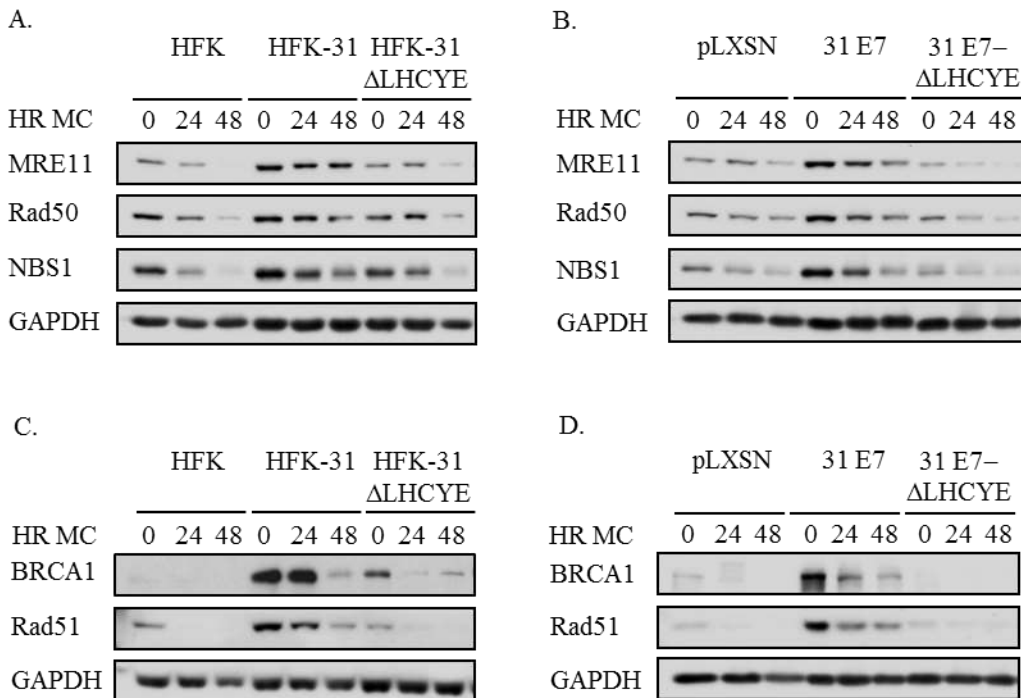
Figure 2.2. Levels of the MRN complex, as well as the homologous recombination proteins BRCA1 and Rad51, are maintained in HPV31 positive cells in a manner dependent on the E7 Rb binding domain. (A) Whole cell lysates were harvested from HFKs, HFKs stably transfected with wild-type HPV31 genomes (HFK-31), or HPV31 genomes with a mutation in the E7 Rb binding domain (HFK-31 ΔLHCYE) at T0 (undifferentiated), as well as after 24 and 48hr differentiation in methylcellulose (MC). Western blotting was performed using antibodies for MRE11, Rad50, and NBS1, with GAPDH serving as a loading control. Western blots shown are representative of five independent experiments across four HFK backgrounds. (B) Whole cell lysates were harvested from HFKs stably transduced with either the empty retroviral vector (pLXSN), wild type HPV31 E7 (pLXSN-31 E7), or an E7 containing a mutation in the Rb binding

domain (pLXSN-31 E7-ΔLHCYE) at T0 (undifferentiated) as well as after 24 and 48hr

differentiation in MC. Western blotting was performed with antibodies targeting MRE11,

Rad50, and NBS1. The blots shown are representative of three independent

experiments across two HFK backgrounds. (C) Whole cell lysates were harvested from

HFKs, HFK-31, and HFK-31 ΔLHCYE cells, both at T0 and after 24 and 48hr

differentiation in MC. Western blot analysis was performed for BRCA1 and Rad51, with

GAPDH serving as a loading control. The data shown is a representative example of

five independent experiments across four HFK backgrounds. (D) Whole cell lysates

were harvested from pLXSN, pLXSN-31 E7, and pLXSN-31 E7-ΔLHYCE cells both prior

to and 24 and 48 hours post differentiation in MC, and Western blot analysis was

performed using BRCA1 and Rad51 antibodies, with GAPDH serving as a loading

control. Shown is a representative Western blot from three independent experiments

across two HFK backgrounds.

Elevated levels of DNA repair factors in HPV31 positive cells cannot solely

be explained by increased transcription: The inactivation of Rb results in the

constitutive activation of a subset of E2F transcription factors termed the activator E2Fs

(E2F1-3) that drive transcription of a number of cellular genes not only involved in

facilitating S-phase entry, but DNA repair as well, including ATM, Chk1, BRCA1, and

Rad51 (6). Previous studies by the Laimins lab showed that E2F2 protein levels are

increased in HPV31 positive cells upon differentiation in a manner dependent on the E7

Rb binding domain (28). E7 may therefore contribute to activation of the DDR, at least in

part, by increasing transcription of key DNA repair genes in an E2F-dependent manner.

To investigate this possibility, we first examined if the protein levels of the other

activator E2Fs, E2F1 and E2F3, are also affected by the E7 ΔLHCYE mutation in

HPV31 positive cells. As shown in Figure 2.3A, the levels of E2F1 and E2F2, but not

E2F3, were increased in HFK-31 cells compared to uninfected HFKs, and this

phenotype was lost in cells containing E7 ΔLHCYE mutant genomes. We next

confirmed these results in cells stably expressing E7 alone. As shown in Figure 2.3B,

while HFKs expressing wild-type E7 exhibited elevated E2F1 and E2F2 protein levels,

this increase was not observed in the E7 ΔLHCYE mutant, similar to results found with

the HFK-31 ΔLHCYE mutant genome lines (Figure 2.3A). Additionally, E2F3 was

regulated in a manner similar to E2F1 and E2F2 in E7-expressing cells (Figure 2.3B),

which was not observed within the context of the HPV genome (Figure 2.3A). Given the

relationship between the E7 Rb binding domain and maintenance of E2F proteins, we

examined the possibility that E7 increases the levels of DNA repair factors at the level of

gene expression.
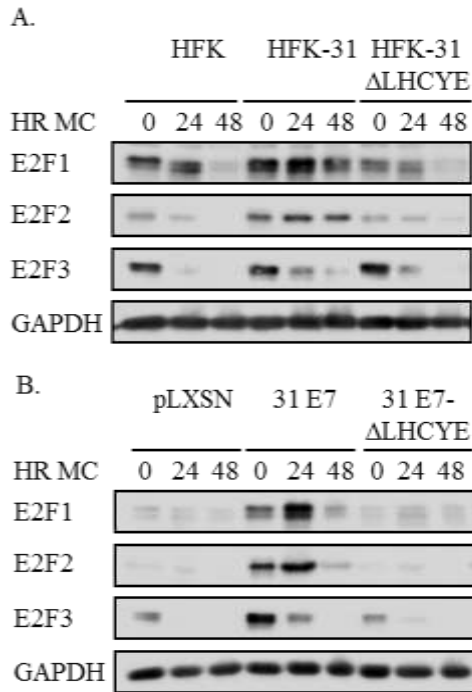
Figure 2.3. The Rb binding domain of E7 is necessary for increased levels of E2F1 and E2F2 in HPV positive and E7-expressing keratinocytes. (A) Whole cell lysates were harvested from HFKs, HFKs containing wild-type HPV31 genomes (HFK-31), or HPV31 genomes containing an E7 Rb deletion mutant (HFK-31 ΔLHCYE) that were undifferentiated (T0) or differentiated for 24 or 48 hours in methylcellulose (MC). Western blot analysis was performed using antibodies to E2F1, E2F2, and E2F3. GAPDH served as a loading control. The Western blots shown are representative of five independent experiments across four HFK backgrounds. (B) Whole cell lysates were harvested from HFKs stably transduced with a retroviral control vector (pLXSN), or vector expressing wild-type HPV31 E7 (pLXSN-31 E7), or an E7 containing a mutation in the Rb (pLXSN-31 E7-ΔLHCYE) that were undifferentiated (T0) or differentiated in methylcellulose for 24 and 48 hours. Western blot analysis was performed as described

in Panel A. Shown are Western blots representative of three independent experiments across two HFK backgrounds.

We first examined the effect of mutating the E7 Rb binding domain on the transcript levels of ATM, Chk2, and Chk1 in HPV31 genome-containing HFKs (Figure 2.4). As shown in Figure 2.4A, HFK-31 cells exhibited no significant changes in transcript levels for ATM, Chk2, or Chk1 in comparison to uninfected HFKs, despite exhibiting substantially increased protein levels (Figure 2.1A). While loss of the E7 Rb binding domain did slightly affect the levels of ATM, Chk2, and Chk1 transcripts, these changes were statistically insignificant ($p>0.05$) (Figure 2.4A). In E7-expressing HFKs, no significant changes were observed for ATM or Chk2 transcripts compared to control cells, however, mRNA levels of ATM and Chk2 were reduced in the E7 ΔLHCYE mutant (Figure 2.4B). For Chk1, E7-expressing cells exhibited a minor, though significant increase in transcript levels compared to HFKs. Similar to ATM and Chk2, Chk1 transcript levels were decreased in the E7 ΔLHCYE mutant cells (Figure 2.4B). These results indicate that while elevated transcription may contribute to the increased protein levels of Chk1, the moderate changes in ATM and Chk2 transcript levels in HFK-31 cells compared to HFKs cannot solely account for the high protein levels observed in HPV31 positive cells. In addition, the decrease in transcript levels observed in the ΔLHCYE mutant cannot fully account for the differences in protein levels observed in these cells and suggest that E7 may also regulate levels of these DDR factors in a post-transcriptional manner.

We next determined if loss of the Rb binding domain affects the mRNA levels of MRN complex components in HPV31 positive cells (Figure 2.4C), as well as E7-

expressing cells (Figure 2.4D) Previously, we reported that in HPV31 E7-expressing

cells, MRE11 gene expression was slightly, though significantly elevated compared to

control cells, with no change in Rad50 and NBS1 transcripts (2). To confirm these

results in HPV31 positive cells and determine if loss of the Rb binding domain affects

expression of MRE11, Rad50, and NBS1, we first examined mRNA levels in HFKs and

HFK-31 cells, as well as in HFK-31 ΔLHCYE cells. As shown in Figure 2.4C, we found

no significant changes in transcript levels for MRE11, Rad50, or NBS1 between HFK

and HFK-31 cells. Similarly, while small reductions in mRNA levels for MRE11, Rad50,

and NBS1 were observed in HFK-31 ΔLHCYE cells when compared to HFK-31 cells,

these changes were not significant. In HFKs expressing HPV31 E7 alone, we observed

no significant changes between wild-type E7-expressing cells and the vector control for

any of the MRN components (Figure 2.4D). The disparity in these data for Mre11 and

our previously published results likely owes to the very small changes observed in both

cases (<2 fold increase in MRE11). Similar to ATM, Chk2, and Chk1, we did observe a

small but significant decrease in transcript levels for both MRE11 and NBS1, but not

Rad50, in cells expressing the E7 ΔLHCYE mutant. Taken together, these data suggest

that transcription may play a minor role in the increase in the MRN components

observed in HPV31 positive cells, however, increased gene expression alone cannot

fully explain the changes observed in MRN protein levels.

Previously, we demonstrated that HPV31 positive cells exhibit increased

transcript levels of BRCA1 and Rad51, with E7 expression alone being sufficient for this

increase (7). To determine if the increase in gene expression requires the E7 Rb

binding domain, we examined BRCA1 and Rad51 mRNA levels in uninfected HFKs, as

well as HFKs containing wild-type HPV31 genomes, or E7 ΔLHCYE mutant genomes

(Figure 2.4E). As shown in Figure 2.4E, transcript levels for BRCA1 and Rad51 were

increased in HFK-31 cells compared to uninfected HFKs, and this increase was lost in

cells expressing the E7 ΔLHYCE mutant. Similar results were observed for E7-

expressing cells (Figure 2.4F), with BRCA1 and Rad51 transcripts being present at a

significantly increased level compared to control cells, as published previously (7). This

phenotype was again lost in the E7 ΔLHCYE mutant (Figure 2.4F), indicating that in

addition to maintenance of BRCA1 and Rad51 protein levels (Figure 2.2C-D), the E7 Rb

binding domain is also required for increased gene expression of BRCA1 and Rad51.

Overall, these data suggest that E7 regulates the levels of a subset of DDR proteins in a

transcription-independent manner (ATM, Chk2, MRN), while conferring a mechanism of

transcriptional dependence, to a certain extent, on other DDR factors (Chk1, BRCA1,

Rad51).
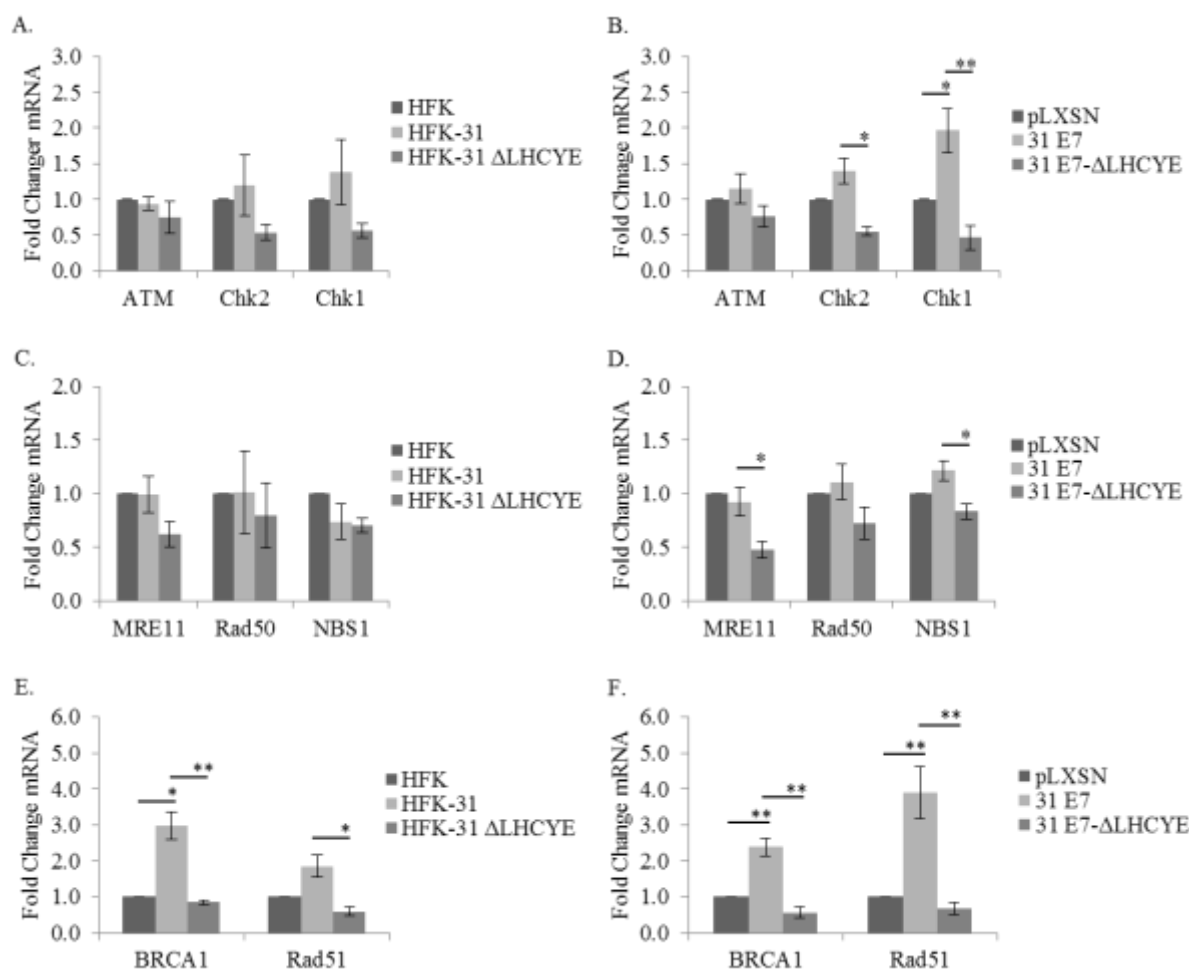
Figure 2.4. The E7 Rb binding domain is required for increased transcript levels of Chk1, BRCA1 and Rad51. RNA was extracted from undifferentiated (A, C, E) HFKs, HFKs maintaining wild type HPV-31 genomes (HFK-31), and HPV-31 genomes containing a deletion in the E7 Rb binding site (HFK-31 ΔLHCYE); (B, D, F) or undifferentiated HFKs retrovirally transduced with either a control vector (pLXSN), wild type HPV31 E7 (pLXSN-31 E7), or the E7-Rb binding mutant (pLXSN-31 E7-ΔLHCYE). Reverse transcription quantitative PCR (RQ-PCR) was performed using gene-specific primers for ATM, Chk2, and Chk1 (A, B), MRE11, Rad50, and NBS1 (C, D), or BRCA1 and Rad51 (E, F). Shown is fold change in transcripts calculated using the $2^{-\Delta\Delta CT}$ method. Values shown are transcript levels relative to either HFK (A, C, E) or pLXSN

(B, D, F), which are set to 1. Error is indicated as +/- the standard error of the mean. Each panel represents the results from four independent experiments derived from two different HFK donors. For all panels, the student's t-test was used to test significance. * $P \leq 0.05$, and ** $P \leq 0.01$.

The E7 Rb binding domain is required for increased protein stability of a subset of DNA repair factors in HPV31 positive cells: Previous studies have demonstrated that the E7 Rb binding domain is required for maintenance of high levels of E2F2 in a post-transcriptional manner (28). In addition, our lab has previously shown that BRCA1 and Rad51 exhibit an increased protein half-life in HPV31 positive CIN612 cells (7). These studies, taken together with the observation that the transcript levels of ATM, Chk2, Chk1, and the MRN components were not substantially altered in HFK-31 ΔLHCYE cells raises the possibility that E7 may contribute to increased DDR protein levels in infected cells through influencing protein stability.

To determine the impact of protein stability on the maintenance of DDR factors in HPV31 positive cells, we first examined the half-life of ATM, Chk2, and Chk1 in HKFs compared to HFK-31 cells using cycloheximide to inhibit protein synthesis. As shown in Figure 2.5A and 2.5B and summarized in Table 2.1, the protein half-lives for ATM, Chk2, and Chk1 in HFKs were 5.8+/-1.3, 7.0+/-0.02, and 4.5+/-0.3 hours, respectively. In contrast, in HFK-31 cells the half-lives of ATM, and Chk2 increased to greater than 12 hours (the longest time point measured). For Chk1, the half-life was also increased in HFK-31 cells (5.8+/-0.5 hours vs. 4.5 +/-0.3 in HFKs), though not to the same extent as ATM and Chk2. To determine if the E7 Rb binding domain was required for the increase in protein stability observed, we examined the half-lives of ATM, Chk2, and

Chk1 in cells containing HPV31 genomes with the ΔLHCYE mutation. Interestingly, in HFK-31 ΔLHCYE cells, the half-lives of ATM, Chk2, and Chk1 were reduced to 4.5+/-0.8, 9.6+/-2.0, and 4.2+/-0.4 hours, respectively, similar to the half-lives observed in HFKs for each protein (Figure 2.5A-B, Table 2.1). These results suggest that high levels of ATM, Chk2, and Chk1 are maintained in HPV31 positive cells, at least in part, through an increase in protein stability.

To determine if E7 expression alone is sufficient for the increase in protein stability observed, we examined the half-life of ATM, Chk2, and Chk1 in HFKs expressing wild-type E7, as well as the E7 ΔLHCYE mutant (Figure 2.5C-D). As shown in Figures 2.5C and 2.5D and summarized in Table 2.2, similar to HPV31 genome-containing lines, the half-life of ATM increased from 4.5+/-0.3 hours in HFKs to >12 hours in E7-expressing cells. In contrast, the half-life of ATM decreased to 4.9+/-0.9 hours in the E7 ΔLHCYE mutant. Similar results were observed for Chk2, with the half-life increasing to >12 hours in HFK-31 cells from 7.5+/-0.5 hours in HFKs. Similar to ATM, Chk2 exhibited a reduced half-life in cells expressing the E7 ΔLHCYE mutant (Figure 2.5C-D, Table 2.1). Since the half-life of Chk2 in the E7 ΔLHCYE mutant cells extended past our time course, we were unable to determine the full effect of loss of the E7 Rb binding domain on Chk2 protein stability. However, the observation that the relative levels of Chk2 decrease in the E7 ΔLHCYE mutant at each time point compared to HFK-31 cells suggests that the E7 Rb binding domain is important for extending the half-life of the Chk2 protein. The half-life of Chk1 was also extended in E7-expressing cells, increasing to 5.6+/-0.8 hours from 3.5+/-0.6 hours in control cells. Similar to ATM and Chk2, the increase in protein stability was lost in cells expressing the E7 ΔLHYCE

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mutant (3.9+/-0.3 hours). Taken together, these data indicate that HPV maintains high levels of ATM, as well as ATR pathway components by increasing protein stability. Furthermore, these data indicate that the increase in half-life observed in HPV31 positive cells occurs in an E7-dependent manner through its Rb binding domain.
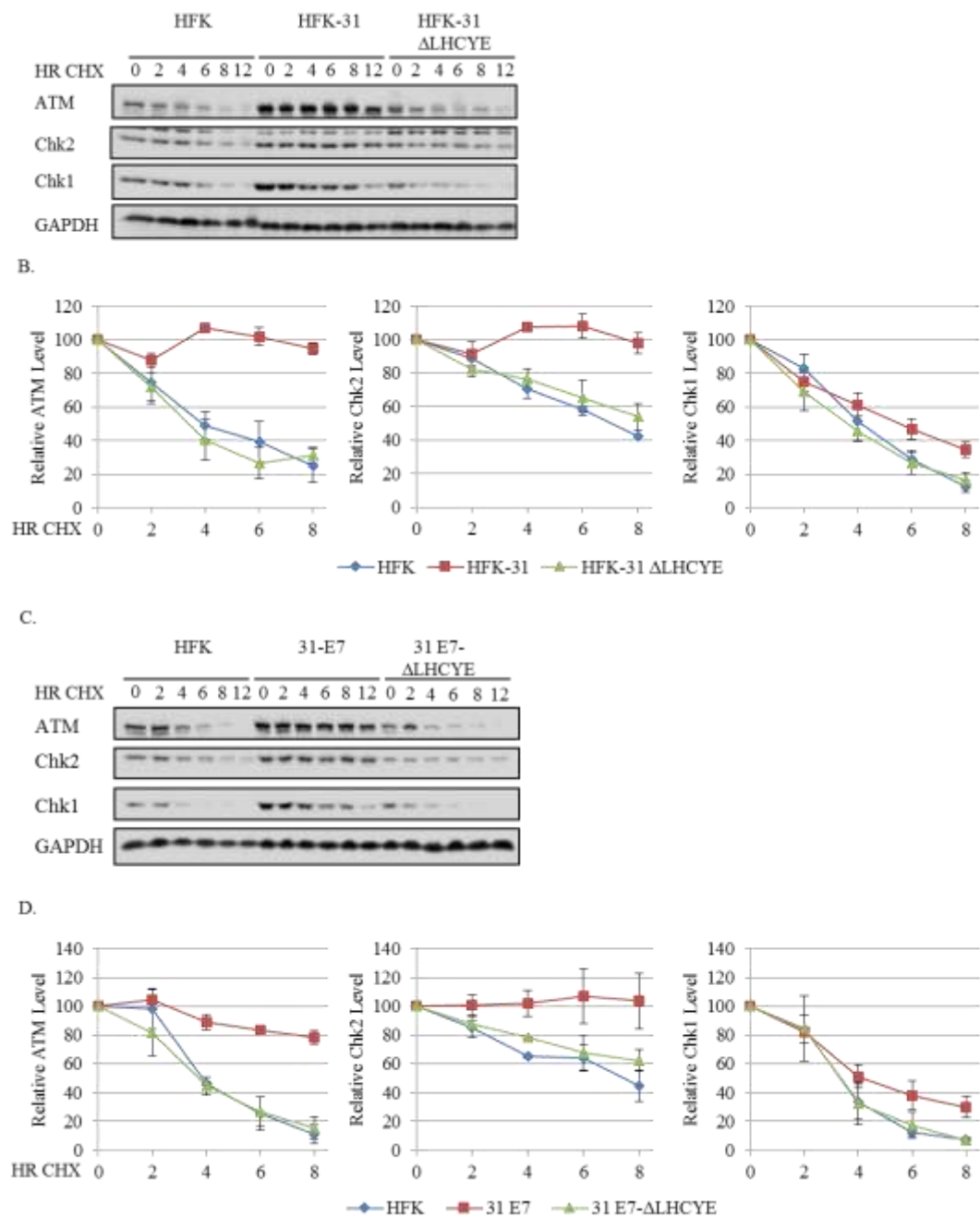
Figure 2.5. The half-lives of ATM, Chk2, and Chk1 are increased in HPV31 positives cells in an E7-dependent manner. (A, B) Uninfected HFKs, HFKs maintaining wild type HPV31 genomes (HFK-31), HPV31 genomes containing a mutation in the E7 Rb binding site (HFK-31 ΔLHYCE), as well as (C, D) HFKs stably transduced with a retroviral vector expressing wild type E7 (pLXSN-31 E7), or E7 with a mutation in the Rb binding domain (pLXSN-31 E7 ΔLHCYE) were treated with 50 □g/ml cycloheximide over 12 hour time course, with whole cell lysates being harvested at the indicated time points. Western blot analysis was performed using antibodies targeting ATM, Chk2, and Chk1, as well as GAPDH, which served as a loading control. (A, B) Shown is representative Western blots from three independent experiments from two different HFK donors. (C, D) Data shown are representative Western blots from three independent experiments from one HFK donor. (B) Graphed are the average protein levels of ATM, Chk2, and Chk1 in HFK, HFK-31, and HFK-31 ΔLHCYE cells over three independent experiments +/- the standard error of the mean. Westerns were digitally imaged using the Bio-Rad Chemidoc MP system, and densitometry was performed with the Biorad ImageLab 5.0 software. Values are shown relative to each T0, which was set to100. (D) Graphed are the average protein levels of ATM, Chk2 and Chk1 in HFKs, pLXSN-E7 and pLXSN E7 ΔLHCYE cells over three independent experiments. Densitometry was performed as described above. Error bars represent +/- the standard error of the mean.

We next determined if HPV31 also maintains components of the MRN complex by increasing protein stability. As shown if Figures 2.6A and 2.6B and summarized in Table 2.1, the half-life of MRE11 was substantially increased from 4.8+/-0.4 hours in

HFKs to >12 hours in HFK-31 cells. In contrast, the half-life of MRE11 in HFK-31

ΔLHYCE cells (4.2+/-0.2 hours) mirrored that found in HFKs. Similarly, the half-life of

Rad50 was increased in HFK-31 cells (>12 hours) compared to HFKs (8.3+/-0.9 hours).

We were unable to determine the effect of the ΔLHCYE mutation on Rad50 protein

stability as the half-life extended beyond our time course (>12 hours) (Figure 2.6B).

However, the observation that the relative levels of Rad50 decreased in the E7

ΔLHCYE mutant at each time point compared to HFK-31 cells suggests that protein

stability is affected by loss of the Rb binding domain. For NBS1, the half-life increased

from 7.1+/-1.1 hours in HFKs to more than 12 hours in HFK-31 cells, and was reduced

to 9.4+/-1.1 hours in HFK-31 ΔLHYCE cells. Similar results for the MRN complex

components were observed in E7-expressing cells (Figure 2.6C-D). As shown in Figure

2.6C and 2.6D and summarized in Table 2.2, the half-lives of MRE11 and NBS1

increased from 3.2+/-0.2 and 5.0+/-0.4 hours in HFKs, respectively, to >12 hours in cells

expressing wild-type E7. In cells expressing the E7 ΔLHYCE mutant, the half-lives for

MRE11 and Nbs1 were reduced to 3.6+/-0.8 and 4.6+/-0.7 hours, respectively, similar to

that found in HFKs (Figure 2.6C-D). Similar to the HPV31 genome containing lines, the

half-life for Rad50 increased to >12 hours in E7-expressing cells compared to 8.4+/-0.3

hours in HFKs. In cells expressing the E7 ΔLHCYE mutant, we were again unable to

calculate the half-life of Rad50 based on the 12-hour time-course utilized. However, at

each time point the relative levels of Rad50 were lower in the E7 ΔLHCYE mutant

compared to wild-type E7-expressing cells, again suggesting that loss of the Rb binding

mutant affects Rad50 stability. Overall, these data suggest that protein stability is a

contributing factor to the maintenance of MRN complex members in HPV31 positive

cells and this regulation occurs through a mechanism requiring Rb binding domain of

E7.

A.

B.

C.

D.
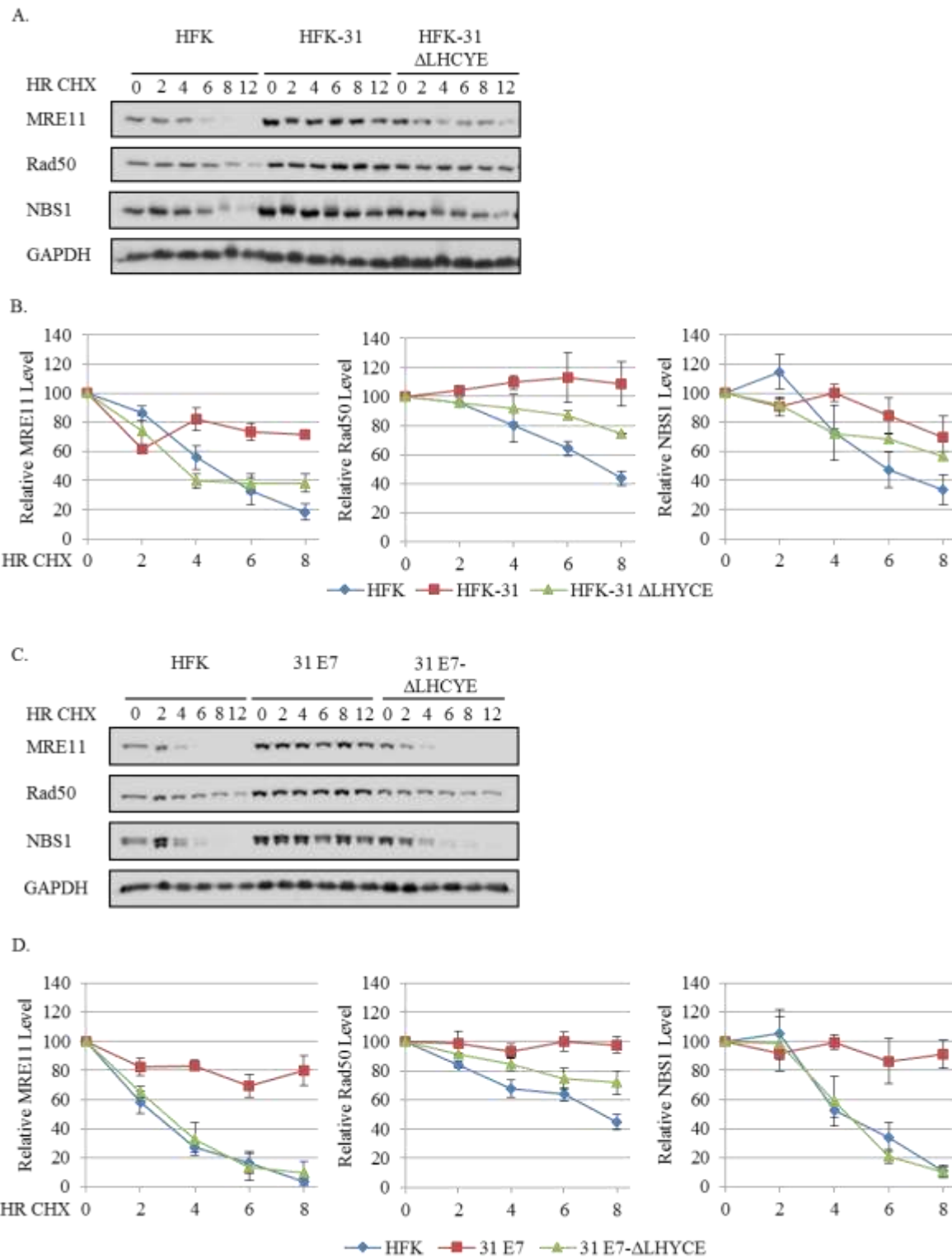
Figure 2.6. The E7 Rb binding domain is necessary for the increased half-lives of MRN complex components in HPV31 positive cells. (A, B) Uninfected HFKs, HFKs maintaining wild type HPV31 genomes (HFK-31), HPV31 genomes containing a mutation in the E7 Rb binding site (HFK-31 ΔLHYCE), as well as (C, D) HFKs stably transduced with a retroviral vector expressing wild type E7 (pLXSN-31 E7), or E7 with a mutation in the Rb binding domain (pLXSN-31 E7 ΔLHCYE) were treated with 50 □g/ml cycloheximide over 12 hour time course, and whole cell lysates were harvested at the indicated time points. Western blot analysis was performed using antibodies to MRE11, Rad50, and NBS1, as well as GAPDH, which served as a loading control. (A, B) Data shown are representative blots from three independent experiments from two different HFK donors, and for (C, D) data shown are representative blots from three independent experiments from one HFK donor. (B, D) Graphed are the average protein levels of MRE11, Rad50, and NBS1 over three independent experiments. Westerns were digitally imaged using the Bio-Rad Chemidoc MP system, and densitometry was performed with the Biorad ImageLab 5.0 software. Values are shown relative to each T0, which was set to100. Error bars represent +/- the standard error of the mean.

As mentioned, we previously reported that BRCA1 and Rad51 exhibit increased protein stability in HPV31 positive CIN612 cells (7). We next wanted to determine if this phenotype was affected by the E7 ΔLHCYE mutation. As shown in Figures 2.7A and 2.7B and summarized in Table 2.1, the half-lives of BRCA1 and Rad51 were significantly elevated, increasing from 2.9+/-0.3 and 5.4+/-0.2 hours in HFKs to 6.0+/-0.5 and 10.2+/-0.3 hours in HFK-31 cells, respectively. Similar to the other DDR proteins examined, this increased stability was lost in HFK-31 ΔLHCYE cells for both

BRCA1 (3.0+/-0.3 hours) and Rad51 (4.6+/-0.2 hours). Similar results were observed in

the E7-expressing cells, as shown in Figures 2.7C and 2.7D and summarized in Table

2.2. We found the half-lives of BRCA1 and Rad51 increased from 3.3+/-0.4 and 5.0+/-

1.4 hours in HFKs to 6.9+/-0.2 and 12.1+/-1.4 hours in E7-expressing cells,

respectively. In the E7 ΔLHCYE mutant cells, the half-lives decreased to levels similar

to that found in HFKs for both BRCA1 (2.6+/-0.1 hours) and Rad51 (5.0+/-0.9). This

data, along with our analysis of BRCA1 and Rad51 gene expression (Figure 2.4E-F),

suggests that BRCA1 and Rad51 are regulated by HPV31 both transcriptionally, as well

as post-transcriptionally via protein stability in an E7-dependent manner. Additionally,

our data indicate that the Rb binding site of E7 contributes to both mechanisms.
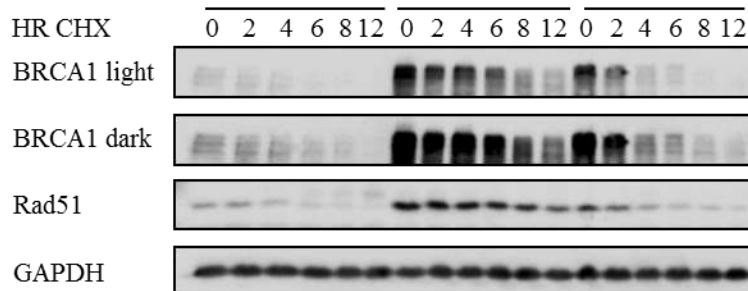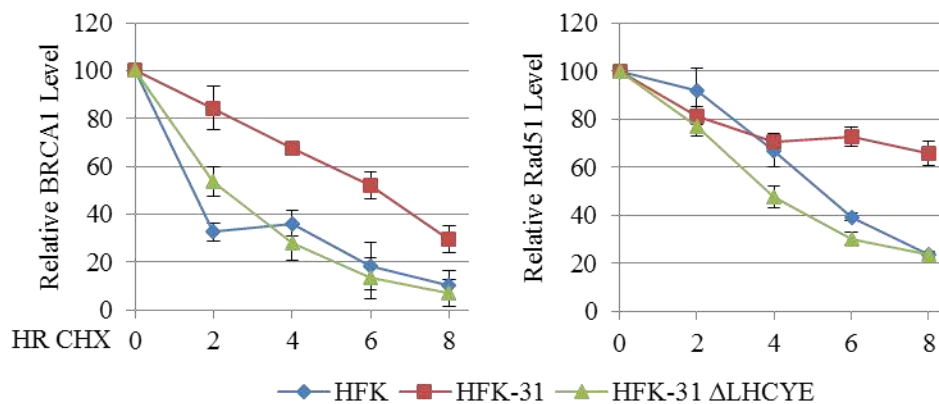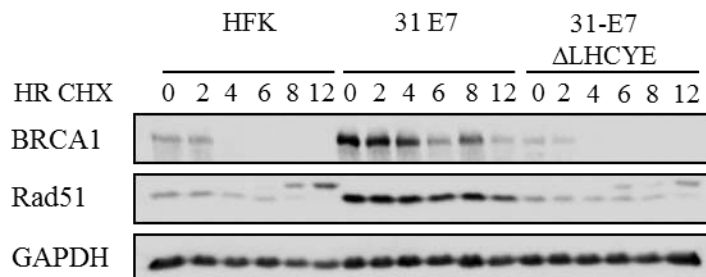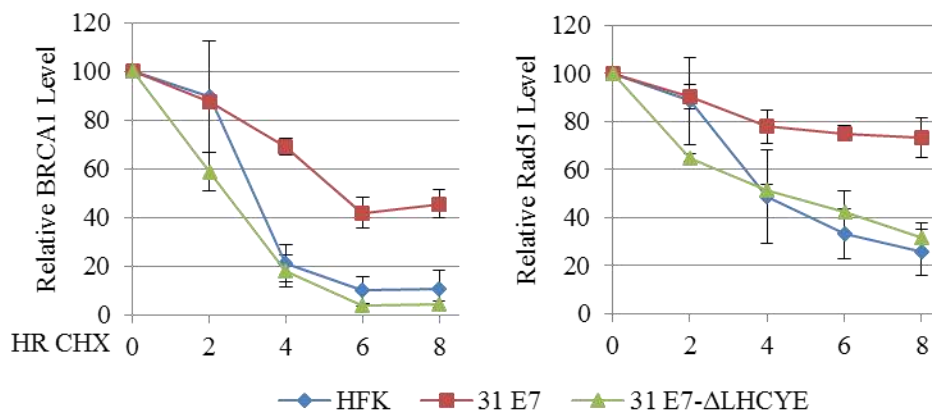
Figure 2.7. Increased protein stability in an E7-dependent manner contributes to the increased levels of BRCA1 and Rad51 in HPV31 positive cells. (A, B) Primary HFKs, HFKs maintaining wild type HPV31 genomes (HFK-31) or mutant HPV31 genomes with a mutation in the Rb binding site (HFK-31 ΔLHCYE), as well as (C, D) pLXSN-E7 and pLXSN E7 ΔLHCYE cells were treated with 50 g/ml cycloheximide over a 12 hour time course. Whole cell lysates were harvest at the indicated times using antibodies to BRCA1 and Rad51, with GAPDH serving as a loading control. (A, B) Data shown are representative blots from three independent experiments from two different HFK donors, and for (C, D) data shown are representative blots from three independent experiments from one HFK donor. (B, D) Graphed are the relative protein levels at each time point, with T0 for each cell line set to 100. Densitometry was performed across three independent experiments using Biorad ImageLab 5.0 software. Error bars represent means +/- standard error.

Protein	HFK	HFK-31	HFK-31 ΔLHCYE
ATM	5.8+/-1.3	>12	4.5+/-0.8
Chk2	7.0+/-0.02	>12	9.6+/-2.0
Chk1	4.5+/-0.3	5.8+/-0.5	4.2+/-0.4
MRE11	4.8+/-0.4	>12	4.2+/-0.2
Rad50	8.3+/-0.9	>12	>12
NBS1	7.1+/-1.1	>12	9.4+/-1.1
BRCA1	2.9+/-0.3	6.0+/-0.5	3.0+/-0.3
Rad51	5.4+/-0.2	10.2+/-0.3	4.6+/-0.2

Table 2.1. Protein half-lives for DDR factors in primary HFKs, HFK-31 cells, and HFK-31 ΔLHCYE cells. Half-lives of the indicated proteins were determined by performing linear regression on values obtained by densitometry from Figure 2.5B,

Figure 2.6B, and Figure 2.7B across three independent experiments from two HFK

backgrounds. Error represents +/- standard error of the mean.

Protein	HFK	31-E7	31-E7 ΔLHCYE
ATM	4.5+/-0.3	>12	4.9+/-0.9
Chk2	7.5+/-0.5	>12	12.3+/-2.7
Chk1	3.5+/-0.6	5.6+/-0.8	3.9+/-0.3
MRE11	3.2+/-0.2	>12	3.6+/-0.8
Rad50	8.4+/-0.3	>12	>12
NBS1	5.0+/-0.3	>12	4.6+/-0.7
BRCA1	3.3+/-0.4	6.9+/-0.2	2.6+/-0.1
Rad51	5.0+/-1.4	12.1+/-1.4	5.0+/-0.9

**Table 2.2. Protein half-lives for DDR factors in primary HFKs, pLXSN 31-E7 cells,

and pLXSN 31-E7 ΔLHCYE cells.** Linear regression was performed on values

obtained by densitometry in Figure 2.5D, Figure 2.6D, and Figure 2.7D to determine the

half-life of the indicated proteins. Shown are the average half-lives across three

independent experiments from one HFK background with the error representing +/-

standard error of the mean.

DISCUSSION

Previous studies demonstrated that activation of both the ATM and ATR DNA

damage response (DDR) pathways are required for the productive replication of HPV31

(16, 31). Expression of E7 alone has been shown to be sufficient for activation of both

ATM and ATR signaling (16, 37). It is well established that E7 plays a central role in

facilitating viral replication by binding and targeting the degradation of Rb, resulting in S-

phase re-entry by a subset of differentiating keratinocytes (3). The introduction of the

HPV31 genome, or expression of HPV31 E7 alone is sufficient to increase the levels of

a broad range of DNA repair factors in primary keratinocytes, including ATM, Chk2,

Chk1, the MRN complex, BRCA1, and Rad51, all of which are required for productive

replication (2, 7, 31, 37). Interestingly, loss of Rb activity has been shown to recapitulate

many of these phenotypes (12, 36), raising the possibility that E7 activates the DDR and

provides DNA repair factors for productive viral replication through its ability to bind and

target Rb for degradation. Consistent with this idea, we have found that the E7 Rb

binding domain is required for maintenance of ATM and ATR activation, as well as the

increased levels of DNA repair factors observed in both HPV31 positive and E7-

expressing cells.

We have found that deletion of the HPV31 E7 Rb binding domain results in

decreased levels of both phosphorylated and total levels of ATM, as well as its

downstream target Chk2. In addition, we have found that this domain is required for

maintenance of total and phosphorylated levels of Chk1, a target of the ATR DNA

damage kinase. The concomitant decrease in total levels along with the

phosphorylated forms of ATM, Chk2, and Chk1 in E7 Rb binding deficient cells

suggests that in addition to inducing DDR activation, E7 contributes to maintenance of

the DDR through increasing total levels of DNA repair factors. Additionally, we show

that while total levels of ATM and Chk2 decrease upon differentiation in HPV31 positive

cells, the phosphorylated forms remain increased, suggesting that the DDR is further

activated during the productive phase of the viral life cycle. In contrast, loss of the Rb

binding domain resulted in a minimal decrease in total levels of DDR factors upon

differentiation, similar to that observed for uninfected HFKs. In cells expressing HPV31

E7 alone, these phenotypes were recapitulated, further highlighting the importance of

the E7 Rb binding domain in the regulation of these DDR factors in HPV31 positive cells.

Our studies demonstrate that loss of the E7 Rb binding site results in reduced levels of all three components of the MRN complex (Mre11, Rad50, and Nbs1). The MRN complex is required for the activation of ATM in response to DNA damage (24, 25, 35). However, we previously published that the MRN complex is not required for ATM activation in HPV31 positive cells, but is required for productive viral replication (2). These results suggest that the decrease observed in ATM phosphorylation upon the loss of the Rb binding domain is not due to decreased levels of MRN components. In addition, we have found that the levels of the homologous repair proteins BRCA1 and Rad51, both of which are also required for productive replication (7), are maintained at high levels in HPV31 positive cells and E7-expressing cells in a manner dependent on the Rb binding domain. Together, these data further suggest that the Rb binding domain is required for HPV to maintain adequate levels of DNA repair factors required for viral DNA synthesis.

The most well-known function of the E7 LXCXE domain is the binding of Rb, resulting in its targeted degradation and the constitutive activation of E2F transcription factors. Based on this function of E7, the simplest explanation of the reduction in total protein levels observed in Rb binding deficient cells is that transcription of DNA repair genes is decreased. In support of this, several of these factors are E2F responsive, including ATM, Chk1, BRCA1, and Rad51 (6). However, while we found that E2F1 and E2F2 protein levels are increased in HPV positive cells in a manner dependent on the Rb binding domain, only Chk1, BRCA1, and Rad51 were significantly affected at the

transcriptional level by the E7 ΔLHCYE mutation, suggesting that the other DNA repair factors examined (ATM, Chk2, and MRN) are regulated primarily in a post-transcriptional manner. In support of this, we found that all of the DDR proteins examined exhibited some level of regulation at the level of protein stability, with five proteins (ATM, Mre11, NBS1, BRCA1, and Rad51) at least doubling their half-lives in HPV31 positive and/or E7-expressing cells compared to uninfected HFKs. Importantly, this increase in half-life was lost in the ΔLHCYE mutant both in the context of the viral genome, as well as in cells expressing E7 alone. Together, these observations suggest that E7 uses a two-pronged approach to elevate levels of DNA repair factors: increasing the transcription of a subset of DNA repair factors and broadly increasing the stability of these proteins. Interestingly, our studies indicate that HPV ensures adequate levels of BRCA1 and Rad51 by targeting both the regulation of gene expression and protein stability.

The mechanism by which E7 increases the protein stability of DNA repair factors in HPV positive cells is currently unclear, although several possibilities exist. E7 has been shown to interact with multiple DDR components, including ATM, Rad50, NBS1, and BRCA1 (2, 31, 46), which may influence the stability of these proteins. Importantly, the interaction of E7 with ATM and NBS1 is lost upon depletion of the Rb binding domain (2, 31), and whether this affects the stability of these proteins will be the focus of future investigations. Another possibility is that E7 influences DDR factor protein stability through effects on protein degradation machinery. E7 has been shown to inhibit the anaphase promoting complex/cyclosome (APC/C) (44), a ubiquitin ligase complex involved in regulation of mitotic progression, as well as the DDR (9). However, whether

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E7-mediated inhibition of this complex occurs in a manner dependent on the Rb binding domain is not known.

We have found that E7 requires the Rb binding domain to maintain ATM, as well as ATR activity. ATM has been shown to affect DDR factor stability both directly through phosphorylation, as well as indirectly through regulation of proteasomal degradation (8). Therefore, loss of ATM activity in the E7 ΔLHCYE mutant may result in decreased stability of downstream targets through either loss of phosphorylation and/or increased degradation. The increase in pChk2 observed upon differentiation in HPV31 positive cells despite decreased total levels supports this possibility. ATM regulates the activity of ubiquitin ligases, including MDM2, through phosphorylation, in turn increasing the stability of p53, as well as Chk2. (29) (23). ATM also regulates the stability of Chk1 through phosphorylation and stabilization of the zinc-finger like protein ZEB1, which interacts with the deubiquitylase USP7 to prevent proteasomal degradation of Chk1 (45). Additionally, p300 is phosphorylated and stabilized by ATM in response to DNA damage, and is in turn required for the stabilization NBS1 (21, 22). Interestingly, p300 has also been shown to bind directly to the LXCXE domain of HPV16 E7 (4), providing a potential direct link between the Rb binding site of E7 and the regulation of NBS1 stability. Furthermore, previous studies have linked Rb inactivation to ATM activation and DSB induction (36, 40, 41), as well as the control of Tip60-dependent acetylation of ATM (40), which is required for ATM autophosphorylation and activation. Importantly, recent studies have shown that Tip60 is required for productive replication of HPV31 (17), presumably through facilitating ATM activation. Determining if the Rb binding domain is required for ATM activation through Tip60, as well as whether ATM activity is

required for maintenance of DDR factor stability in HPV31 positive cells will be important areas of future investigation.

In addition to increasing protein stability, our studies suggest that E7 elevates transcription of a subset of DNA repair genes (Chk1, Rad51, and BRCA1) in a manner dependent on the Rb binding domain, though the mechanism by which this occurs is unclear. Previous studies demonstrated a requirement for the STAT5 transcription factor in the activation of both the ATM and ATR pathways in HPV31 positive cells (16, 18). The activation of STAT5 was shown to be E7-dependent and may require the Rb binding domain. STAT5 knockdown decreased the total levels of ATM, Chk2, BRCA1, and Rad51 in HPV31 positive cells, while only affecting the phosphorylated levels of ATR and Chk1 (16, 18). However, whether the decrease in ATM, Chk2, BRCA1, and Rad51 is regulated transcriptionally or post-transcriptionally is unclear. Additionally, the expression of both BRCA1 and Rad51 is known to be regulated in manner dependent on E2F transcription factors (6), opening the possibility that E7 increases their transcription through its ability to inactivate Rb or its related pocket proteins p107 and p130. Identifying the mechanism(s) by which E7 regulates the transcription of these DNA repair factors will be important to further understand how HPV manipulates DNA damage signaling to facilitate viral replication.

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CHAPTER 3: ATM KINASE ACTIVITY IS NECESSARY FOR THE MAINTENANCE OF G$_2$/M CHECKPOINT PROTEINS IN DIFFERENTIATED HPV POSITIVE KERATINOCYTES

OVERVIEW

Human papillomaviruses (HPV) productively replicate their genome in the upper layers of the stratified epithelia while host keratinocytes are arrested in G$_2$. Levels of numerous proteins regulating mitotic entry from G$_2$ are increased in HPV positive cells and exhibit post-translational modifications and localization consistent with activation of the G$_2$/M checkpoint. While it has been shown that the expression of the HPV E7 protein is sufficient to recapitulate these phenotypes in organotypic raft cultures, the underlying mechanism enabling G$_2$ arrest in HPV infected cells is largely unexplored. Here, we demonstrate that the activity of the ATM kinase is necessary for increased levels of G$_2$/M regulating proteins upon differentiation. Taken with the fact that ATM kinase activity is also needed for productive replication, these data suggest that ATM functions in HPV infected cells to create an environment conducive to productive replication by inducing the G$_2$/M checkpoint.

INTRODUCTION

Human papillomavirus (HPV) infection is one of the most common sexually transmitted infections in the world (10). Infection with a subset of HPVs, termed the high-risk HPVs, has been found to be the causative agent of nearly all cervical cancer cases (32). High-risk HPVs infect the keratinocytes of the stratified epithelium, and

HPV's life cycle is tied directly to the differentiation state of the host cell. Infection

begins when an HPV virion gains access to the basal layer of the epithelium through a

small wound called a microabrasion (26). HPV then infects basal keratinocytes to begin

early gene expression prior to replication of its genome up to 50 copies per cell. From

there, HPV replicates its DNA synchronously as the host keratinocyte divides. When a

basal cell divides, one daughter cell will move upwards in the epithelium and begin the

process of differentiation. Differentiation triggers late gene expression and the onset of

the productive phase of the viral life cycle. Upon the initiation of differentiation,

uninfected cells exit the cell cycle while HPV infected keratinocytes re-enter S-phase

and replicate their DNA (5). After host DNA has been replicated, cells then enter a state

resembling G_2 arrest (3, 6, 29). It is only then that HPV will begin the productive

replication of its genome, termed amplification, increasing the number of HPV genomes

per cell to over 1000 (5). After amplification is complete, infected keratinocytes exit the

cell cycle, assemble mature virions, and subsequently release progeny.

Ataxia-telangiectasia mutated (ATM) is a serine-threonine kinase responsible for

coordinating the cellular response to double stranded DNA breaks (DSBs) by

phosphorylating hundreds of downstream targets to induce DNA repair, cell cycle arrest,

and in the event DNA repair fails, apoptosis (27). During HPV infection, the levels of

numerous proteins in the ATM pathway are increased (23). Pharmacological inhibition

of the ATM kinase or its downstream target checkpoint kinase 2 (Chk2) is sufficient to

block amplification, as is the disruption of the MRN complex (the DNA damage sensor

responsible for activating ATM) and knockdown of the homologous repair proteins

BRCA1 and Rad51 (2, 4, 23). Taken together with the fact that these factors are known

to be recruited to viral replication centers (2, 14), these findings suggest that HPV may activate the ATM pathway in part to utilize DNA repair factors in the replication of its DNA. However, the role of ATM activity in increasing the levels of these factors has not been examined. Half-lives of these DNA repair factors are greatly increased in HPV infected cells and ATM activation has been shown to stabilize a subset of these factors outside the context of infection (17-19, 31). Thus, it is possible that ATM activity functions to increase levels of DNA repair factors necessary for viral replication.

Entry into mitosis from G_2 is regulated by the maturation or mitosis promoting factor (MPF). MPF is a complex of two proteins, cyclin B and cyclin dependent kinase 1 (Cdk1). In a cell with undamaged DNA, levels of cytoplasmic cyclin B rise sharply in G_2 and bind Cdk1. In order to trigger mitotic entry, cyclin B and Cdk1 are imported to the nucleus and Cdk1 is activated by the phosphatase cdc25c which removes inhibitory phosphorylation marks on Cdk1 (24). During DNA damage, ATM phosphorylates and activates Chk2, which in turn phosphorylates the cdc25c phosphatase, inactivating it. Inactivation of cdc25c leads to the accumulation of inhibitory phosphorylation marks on Cdk1, arresting the cell cycle (28). During HPV infection, the levels of cyclin B, Cdk1, cdc25c, as well as Myt1 and Wee1 (the kinases responsible for inhibiting Cdk1) are all increased relative to uninfected keratinocytes (3). Importantly, analysis of the localization and posttranslational modifications of these factors suggest that the G_2/M checkpoint is activated in the upper layers of the epithelium in which HPV replicates its genome (3, 29). Specifically, the MPF exhibits cytoplasmic localization and Cdk1 shows increased levels of inhibitory phosphorylation. Additionally, cdc25c is inhibited while total levels Myt1 and Wee1 are increased relative to uninfected cells (3). Together,

these data suggest HPV promotes cell cycle arrest in G_2 in order to facilitate genome amplification.

HPV E7 is a major oncoprotein linked to HPV related cancer. Within the context of the viral life cycle, HPV E7 is most famous for its degradation of the pocket protein Rb to release E2F transcription factors and drive the cell into S-phase. However, E7 has been shown to have a number of other functions as well (25). Expression of E7 alone has been shown to recapitulate both ATM activation and increase the expression of proteins regulating the G_2/M checkpoint (3, 23). Interestingly, deletion of the Rb binding domain of E7 completely ablates both phenotypes (3, 19). Taken with the fact that ATM is a key regulator of the G_2/M checkpoint (27), we hypothesized that HPV may induce G_2/M arrest through E7's activation of ATM. In this study, we begin to examine this question through the use of the small molecule inhibitor of ATM kinase activity, KU-55933. We demonstrate that inhibition of ATM is sufficient to lower levels of G_2/M checkpoint proteins in differentiated HPV31 positive keratinocytes. In contrast, we found that inhibition of ATM kinase activity had no effect on levels of DNA repair factors, with the exception of Rad51. These data suggest that ATM functions to promote G_2/M arrest in HPV infected keratinocytes in order to create an environment conducive to viral replication.

MATERIALS AND METHODS

Cell Culture: CIN612 (9E) cells are a clonal cell line derived from a CIN1 cervical lesion, previously shown to stably maintain HPV31 epitomes (16).Cells were grown in E-medium supplemented with 5 ng/ml mouse epidermal growth factor (EGF) and J2 3T3 fibroblasts growth arrested with mitomycin C, as described previously (30).

Prior to harvesting either protein or DNA, J2 feeder cells were removed by treating with 1mM EDTA in phosphate buffered saline (PBS) for 5 minutes, then washing twice with PBS.

Differentiation in High Calcium Medium: CIN612 cells were differentiated for 72 hours in high calcium media as previously described (23) . Briefly, cells were harvested during log phase growth at approximately 90% confluency (0 hr). The remaining plates were washed with PBS and serum starved in keratinocyte growth media with supplements for 16 hours (KGM; Lonza). Cells were washed again with PBS and then incubated in high calcium medium (1.8mM $CaCl_2$ KGM without supplements) for 72 hours. The media was removed at 48 hours and replaced with fresh high calcium media. DNA and protein was harvested at each time point.

Drug Treatment: KU-55933 was obtained from Selleckchem. For undifferentiated samples (0 hr), cells were treated with 10 μM KU-55933 or DMSO 24 hours prior to harvest. For differentiated samples (72 hr), cells were treated with either 10 μM KU-55933 or DMSO upon the addition of high calcium media. 10 μM KU-55933 or DMSO was added again when the media was changed at 48 hours.

Western Blot Analysis: Whole cell lysates were taken by suspension in RIPA lysis buffer supplemented with PhoSTOP phosphatase inhibitor (Roche) and Complete Mini protease inhibitor (Thermo Scientific). The concentration of protein in each lysate was determined using the Bio-Rad protein assay (Bio-Rad). Equal amounts of protein for each sample were then loaded, separated by size via SDS-page, and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). The following primary antibodies were used during western blotting: phospho-ATM Ser1981, NBS1

(Abcam); ATM (Bethyl laboratories); cdc25b, phospho-cd25c Ser216, phospho-Cdk1 Thr14, phospho-Cdk1 Tyr15, phospho-Cdk1 Thr161, Cdk1, phospho-Chk2 Ser68, Chk2, cyclin A, Myt1, Wee1, (Cell Signaling Technology); cyclin B1, cdc25c, GAPDH, Rad51 (Santa Cruz); MRE11, Rad50, and BRCA1 (GeneTex). Horseradish peroxidase (HRP)-conjugated secondary antibodies used were: anti-rabbit (Cell Signaling Technology) and anti-mouse (GE-life sciences). Enhanced chemiluminescence Clarity (Bio-Rad) substrate was used to develop blots, and images captured using the ChemidocMP imaging system and Imagelab 5.0 software (BioRad).

Southern Blot Analysis: Isolation of DNA and Southern blotting has been described previously (9). Briefly, cell pellets were suspended in lysis buffer containing 400mM NACL, 10mM Tris pH 7.5, 10 mM EDTA, and 30 μL of 20% Sodium Dodecyl Sulfate (SDS). Samples were then incubated overnight at 37°C with 15 μL of 10 mg/ml proteinase K. DNA was extracted using phenol-chloroform and precipitated with 200 proof ethanol. The restriction enzymes HINDIII and BAMHI (New England Biolabs) were used to digest total DNA to produce HPV genomes that were cut either once or not at all, respectively. DNA samples then underwent electrophoresis for 16 hours at 40V in a 0.8% agarose gel and vacuum transferred to a positively charge nylon membrane (Immoblin-NY+; EMD Millipore). DNA was fixed to membranes using UV irradiation and hybridized to P32 labeled probes derived from HPV31 plasmids.

RESULTS

Inhibition of ATM kinase activity has no effect on the levels of DDR factors in HPV31 positive cells: To determine the function of ATM kinase activity in the productive Human papillomavirus (HPV) life cycle, we utilized the small molecule

inhibitor of ATM kinase activity KU-55933. Our lab has previously used this inhibitor to study the effect of ATM inhibition on HPV infection, demonstrating that ATM kinase activity is necessary for genome amplification but not for episomal maintenance (23). HPV31 positive CIN612 cells were treated with Ku-55933 either for 24 hours prior to differentiation or for 72 hours during differentiation in high calcium media, shown to initiate the productive phase of the HPV life cycle. Whole cell lysates were harvested and the levels of DNA repair factors were then examined by western blot analysis. As can be seen in Figure 3.1A, KU-55933 treatment decreased the phosphorylation of ATM at Serine1981, a marker of activated ATM, both prior to and after 72 hours differentiation in calcium medium. Phosphorylated levels of the ATM target Chk2 were also decreased upon KU-55933 treatment. KU-55933 treatment did not affect total levels of ATM or Chk2. Furthermore, levels of the differentiation marker involucrin were not affected by KU-55933 treatment suggesting differentiation proceeded normally. This phenotype is consistent with previous studies utilizing KU-55933, and indicates successful inhibition of ATM kinase activity (23). Additionally, KU-55933 treatment blocked amplification of the HPV31 genome (Figure 3.1B), as previously reported (23).

Many DNA repair proteins previously shown to be necessary for amplification are also the targets of ATM phosphorylation, including MRE11, Rad50, NBS1, and BRCA1 (11-13, 20). We previously showed that the stability of these proteins is greatly upregulated in HPV31 positive cells (19). Given that ATM activation has been shown to regulate the stability of its downstream targets (7), we next wanted to determine if the loss of amplification of the HPV31 genome could be explained by decreased levels of DNA repair factors required for productive replication. To examine this possibility, we

determined the total protein levels of the MRN complex proteins MRE11, Rad50, and

NBS1 as well as the homologous repair factors BRCA1 and Rad51 in response to

inhibition of ATM activity. As shown in Figure 3.1C, inhibition of ATM kinase activity had

no effect on the total levels of MRE11, Rad50, NBS1, or BRCA1 in HPV31 positive

CIN612 cells over our time course. In contrast to this, Rad51 levels were decreased

slightly, though only after differentiation for 72 hours in calcium media. Together these

data suggest that while ATM kinase activity may play a role in increasing total protein

levels of Rad51 specifically, it cannot account for the broad increase in DNA repair

factor levels seen in HPV infected cells.
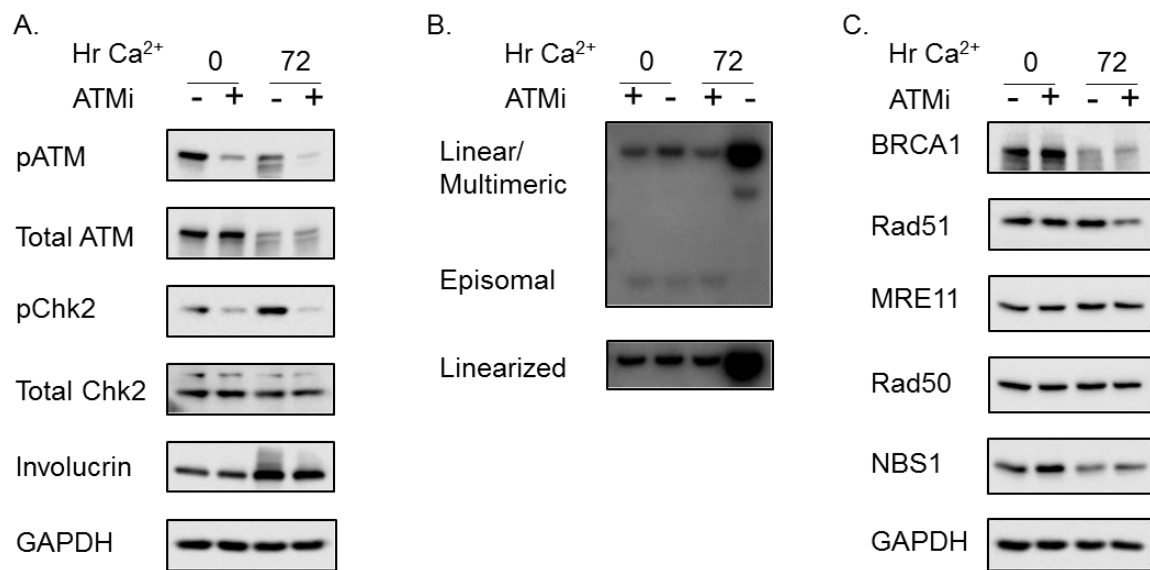
**Figure 3.1. Levels of the MRN complex, as well as the homologous repair protein
BRCA1, are not affected by inhibition of the ATM kinase.** (A) Western blot analysis
was performed on whole cell lysates from HPV31 positive CIN612 cells harvested prior
to (0) and 72 hours after differentiation in high calcium. Cells were treated with either
the ATM kinase inhibitor KU-55933 (ATMi) or DMS0 as a vehicle control, as indicated.

Western blot analysis was performed using phospho-specific antibodies to determine the activation of ATM (Serine 1981) and Chk2 (Thr68), as well as total protein levels. The differentiation marker involucrin was used to confirm differentiation. GAPDH was used as a loading control (B) DNA was harvested from undifferentiated (0) CIN612 cells or those differentiated in high calcium media for 72 hours. Southern blot analysis was performed using an HPV31 specific probe to determine relative levels of HPV31 replication. (C) Western blot analysis was performed as in (A), utilizing antibodies specific for BRCA1, Rad51, MRE11, Rad50, or NBS1. GAPDH served as a loading control.

ATM kinase activity is required to maintain levels of G_2/M checkpoint proteins upon differentiation: Previous studies have demonstrated that HPV amplifies its genome when the host keratinocyte is arrested in G_2 after the host DNA has finished replication (3, 6, 29). Additionally, the levels of several proteins involved in the G_2/M checkpoint are increased in HPV infected cells and HPV E7 expressing cells (3). Given that the ATM pathway is a key activator of the G_2/M checkpoint outside the context of infection (8), we wanted to determine if ATM played a role in arresting cells in G_2. To test this hypothesis, we determined if inhibition of ATM kinase activity by KU-55933 treatment was sufficient to reduce levels of G_2/M proteins in HPV positive cells. We first examined the levels of cyclin B and Cdk1, as these two proteins together regulate mitotic entry by forming a complex known as the maturation or mitosis-promoting factor (MPF). As seen in Figure 3.2A, inhibition of ATM activity decreased total levels of Cdk1, but not cyclin B, after differentiation in calcium. No effect was seen prior to differentiation on either protein. In addition to the binding of cyclin B, activity of Cdk1 is

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regulated by three phosphorylation sites. Phosphorylation on Threonine 14 and Tyrosine 15 serve to inhibit Cdk1 activity, while Cdk1 is activated by phosphorylation at Threonine 161. When the levels of the three regulatory phosphorylation sites of Cdk1 were examined, similar decreases in levels were found in all three cases. Together, these data suggest that ATM kinase activity is necessary for the increased levels of Cdk1, but not cyclin B, in HPV31 positive cells upon differentiation.

During DNA damage, activity of the MPF is largely regulated by the cdc25b and cdc25c phosphatases. During normal cycling, cdc25b and cdc25c remove the inhibitory phosphorylation marks from Cdk1, allowing MPF to become active and drive cells into mitosis from G_2. The activity of cdc25c is itself inhibited by phosphorylation of Serine 216 by the ATM target Chk2 during DNA damage, thus preventing MPF activity by causing the accumulation of inhibitory post translational modifications. Cdc25b's activity is more specialized, and is inhibited by phosphorylation of p38 and Chk1 in response to UV irradiation (28). As with Cdk1, both the phosphorylated and total levels of cdc25c are upregulated in HPV infected and E7 expressing cells, while cdc25b levels have not been examined (3). We next wanted to determine if inhibition of ATM activity by KU-55933 decreased levels of cdc25b and cdc25c in HPV31 positive cells. As shown in Figure 3.2B, while inhibition of ATM had no effect on either phosphorylated or total cdc25c levels prior to differentiation, both were reduced upon differentiation in calcium. An identical phenotype was observed for total levels of cdc25b. Together these data indicate that ATM activity is necessary to maintain high levels of cdc25 proteins upon differentiation in HPV31 positive cells.

Levels of Myt1 and Wee1, the kinases responsible for conferring inhibitory phosphorylation marks on Cdk1, are also increased in HPV positive and E7 expressing cells (3). Given the observation that Cdk1 and cdc25 levels are decreased upon inhibition of the ATM kinase, we next examined the levels of Myt1 and Wee1. As see in Figure 3.2C, levels of Myt1, but not Wee1, were reduced in both unidifferentiated and differentiated HPV positive cells by ATM inhibition. As seen with Cdk1 and cdc25 levels, ATM had no effect prior to differentiation. Together, these data suggest that the ATM kinase is required for increased levels of key proteins regulating the G_2/M checkpoint in differentiating HPV31 positive cells.
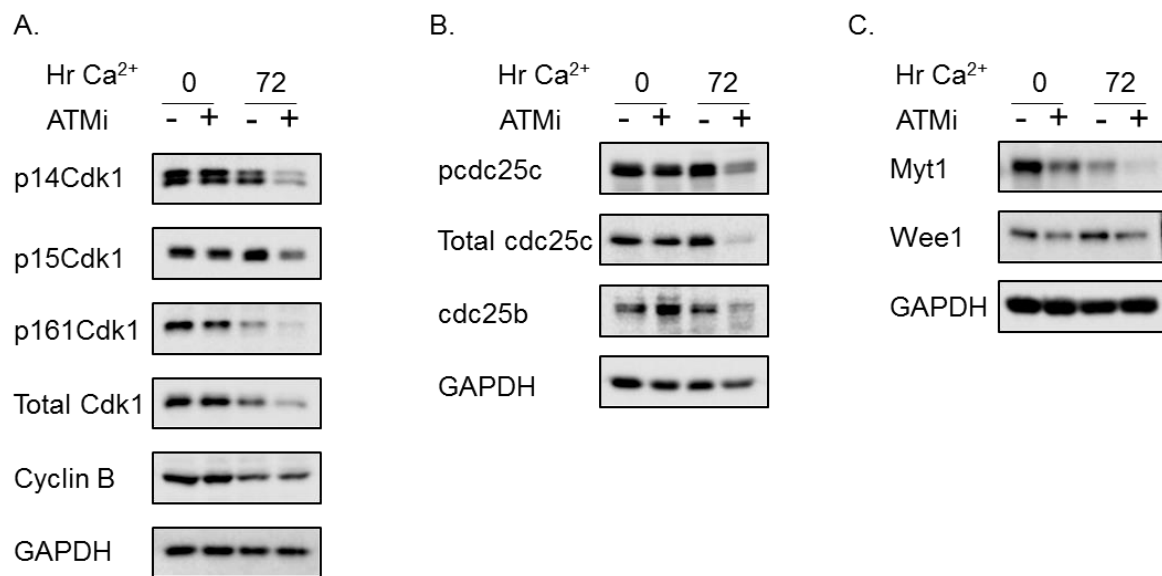
Figure 3.2. Inhibition of the ATM kinase results in decreased levels of G_2/M regulatory proteins in HPV31 positive cells. (A-C) Whole cell lysates from HPV31 positive CIN612 cells were harvested either before (0) or 72 hours after differentiation was induced with high calcium media. Cells were treated with DMSO vehicle control or the KU-55933 (ATMi) inhibitor of ATM kinase activity, as indicated. (A) Western blot analysis was performed with phospho-specific antibodies targeting Threonine 14,

Tyrosine 15, or Threonine 161 of Cdk1, as well as total Cdk1 and cyclin B. (B) Western

blot analysis was performed using a phospho-specific antibody for Serine 216 of

cdc25c, as well as antibodies targeting total cdc25c and cdc25b. (C) Western blot

analysis was performed using antibodies Myt1 and Wee1. GAPDH was used as a

loading control in all panels.

DISCUSSION

It has been previously established that Human papillomavirus (HPV) productively

replicates its genome when cells are arrested in G_2, as evidenced by the high levels of

cytoplasmic cyclin B in cells amplifying viral DNA (29). Furthermore, HPV infected

keratinocytes exhibit elevated levels of G_2/M regulatory proteins such as cyclin B,

cdc25c, Cdk1, Myt1, and Wee1. When it has been examined, all of these proteins have

post-translational modifications and localization consistent with arrest in G_2. Expression

of wild type E7 is sufficient to recapitulate these phenotypes, while expression of an E7

lacking its LXCXE Rb binding motif is not (3). To date, the underlying mechanism of

how E7 is able to induce G_2/M arrest within cells replicating HPV DNA has not been

explored. Outside the context of infection, the ATM kinase, through its downstream

target Chk2, can induce G_2/M arrest in response to DNA damage (27). HPV infected

cells have increased levels of activated ATM and Chk2, and inhibition of the kinase

activity of either protein inhibits productive replication (23). Similar to the arrest of HPV

infected cells in G_2, this phenotype can be recapitulated by the expression of wild type

E7, but not by an E7 lacking its Rb binding domain (19). Given that ATM is an

established inducer of the G_2/M checkpoint and the phenotypic similarities between

G_2/M checkpoint induction and ATM activation in HPV infected cells, we hypothesized

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that ATM activity is necessary for productive HPV replication in part because it ensures the induction of G_2/M arrest during the HPV life cycle.

It was first important to determine if inhibition of ATM had any effect on the levels of DNA repair factors in HPV31 positive cells. The half-lives of many DNA repair factors are increased in HPV31 positive cells. Given that ATM itself has been shown to modulate the stability of some of its downstream targets outside the context of infection (7, 19), it was possible that inhibition of ATM kinase activity could result in decreased levels of DNA repair factors in HPV31 positive cells. However, we found that inhibition of the ATM kinase had no effect on MRE11, Rad50, NBS1, and BRCA1 levels. In contrast, Rad51 levels were mildly decreased. Thus, while ATM kinase activity does play a role in regulating Rad51 levels in HPV infection, it cannot account for the broad increase in DNA repair factor levels seen in HPV infected cells.

We next examined the effect of ATM kinase inhibition on the levels of proteins regulating the G_2/M checkpoint in HPV infected cells. We first examined the levels of cyclin B and Cdk1, the two proteins that make up the maturation or mitosis promoting factor (MPF), which directly controls entry into mitosis from G_2 (24). We found that inhibition of the ATM kinase in HPV positive cells decreased the levels of Cdk1, but not cyclin B, after differentiation in high calcium media. No effect was seen prior to differentiation on either cyclin B or Cdk1. Additionally, the levels of Cdk1 phosphorylated at Threonine 14 and Tyrosine 15 (the inhibitory phosphorylation sites of Cdk1) as well as Threonine 161 (the activating phosphorylation site of Cdk1) were decreased to a similar extent. Similarly, the levels of the Cdk1 regulating phosphatases cdc25b and cdc25c were reduced in HPV infected cells when ATM was inhibited, but only after

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differentiation in high calcium media. Myt1, the kinase responsible for phosphorylating

Cdk1 at Threonine 14, was similarly reduced upon differentiation. In contrast to this,

ATM inhibition had no effect on Wee1, the kinase responsible for phosphorylating Cdk1

at Tyrosine 15. Together, these data indicate that ATM activity is necessary for

increased levels of G_2/M checkpoint proteins in differentiating HPV infected cells.

Given the preliminary nature of the data presented here, a number of additional

experiments are needed to fully explore the implications of our findings. First and

foremost, it is important to demonstrate that G_2 arrest is actually being inhibited in the

upper layers of the stratified epithelium, as a reduction in the levels of proteins

regulating the G_2/M checkpoint does not necessarily demonstrate that arrest does not

occur. To demonstrate this more thoroughly, we will determine if ATM inhibition alters

the localization of cyclin B and Cdk1 in organotypic raft cultures. If ATM inhibition

prevents arrest in G_2, we would expect to see increased levels of nuclear cyclin B and

Cdk1 in suprabasal keratinocytes. These data would demonstrate that MPF is no longer

inhibited and that the G_2/M checkpoint is no longer active.

Because of ATM's large number of downstream targets (27), we would next

examine the exact mechanism ATM uses to induce the G_2/M checkpoint. The most

likely hypothesis is that ATM activates the G_2/M checkpoint through Chk2, the kinase

ATM activates in order to induce G_2 arrest in uninfected cells. Despite the seemingly

obvious nature of this experiment, demonstrating that Chk2 inhibition recapitulates the

effects of ATM inhibition on the G_2/M checkpoint is critical, as it begins to separate oG_2

arrest from other ATM dependent functions. To further support the idea that arrest in G_2

is a specific function of ATM activation needed for productive replication,

pharmacological inhibition of either Myt1 or Wee1 could be used. If inhibition of either kinase blocks productive replication in addition to G_2 arrest, it would suggest that arrest in G_2 is specifically needed for productive HPV replication. Similarly, expression of dominant negative forms of cdc25c or Cdk1 could be used to further support this conclusion. Together, these experiments would demonstrate that G_2 arrest by ATM activation is needed for productive replication of the HPV genome.

In addition to ATM, the ATR single stranded DNA break repair pathway is activated during HPV infection, and inhibiting the kinase activity of ATR or its downstream target Chk1 also blocks productive replication (1, 15). During DNA damage in uninfected cells, there is considerable cross talk between the ATM and ATR pathways (22). Both Chk1 and Chk2 are implicated in the induction of the G_2/M checkpoint, with some reports suggesting that ATR-Chk1 signaling plays a dominant role in certain contexts (21). Thus, the ATR pathway may play a role in activation of the G_2/M checkpoint in HPV infected cells. In the future, it would be interesting to examine the consequences of ATR and Chk1 inhibition on G_2/M arrest, as it is possible that HPV utilizes both pathways to efficiently arrest cells in G_2 in order to facilitate the replication of its genome.

In summary, these data suggest that ATM kinase activity in HPV infected cells is required to increase levels of proteins that regulate the G_2/M checkpoint upon initiation of the productive life cycle by epithelial differentiation. In contrast, ATM activity was not found to be necessary to increase levels of DNA repair factors of the ATM pathway, with the notable exception of Rad51. While these data do not preclude ATM from having additional functions, they do suggest that ATM is required for productive replication of

the HPV genome in part because it induces arrest in G_2. In the future, we hope to more conclusively demonstrate ATM's role in inducing G_2 arrest in HPV infected cells. Determination of the role of ATM, and eventually ATR, pathways in G_2 arrest will allow the development of a more complete model of cell cycle manipulation by HPV, and may aid in the identification of novel interventions to prevent productive HPV replication.

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CHAPTER 4: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

GENERAL SUMMARY

This dissertation examined the activation of the ATM-mediated DNA damage response (DDR) during human papillomavirus (HPV) infection. Previous studies established two important conclusions upon which this dissertation is based. First, papers from the Moody and Laminins labs established that levels of many DDR proteins are increased in HPV positive cells, recruited to viral replication centers, and are required for productive replication (1, 2, 7, 14, 15, 31). Additionally, several studies from the Chow lab showed that productive replication occurs when infected keratinocytes are arrested in G_2 (4, 9, 46). Critically, expression of the HPV E7 protein alone is sufficient to recapitulate these phenotypes.

I expanded on these observations by demonstrating that the Rb binding domain of HPV31 E7 is necessary to increase the levels of DDR proteins in HPV31 positive cells. I also demonstrated that HPV31 increases the levels of these factors by greatly extending their half-lives rather than solely through increased transcription. Additionally, I showed that ATM kinase activity is dispensable for increased levels of DNA repair factors in HPV31 positive cells. In contrast to this result, I found that inhibition of the ATM kinase decreased the levels of proteins regulating the G_2/M checkpoint HPV positive cells undergoing differentiation, suggesting that ATM activates the G_2/M checkpoint during HPV infection. In this final chapter, I will describe these observations in the wider context of HPV infection,

detailing a model of how E7 exploits the ATM pathway in order to facilitate the replication of HPV DNA. I will also describe the potential implications of these findings on HPV induced oncogenesis.

THE FUNCTION OF THE E7 RB BINDING DOMAIN IN THE ACTIVATION OF THE ATM PATHWAY DURING HPV INFECTION

Previous studies firmly established that the expression of HPV E7 is sufficient to activate the DDR by increasing the levels of numerous DNA repair factors (1, 2, 7, 14, 15, 31). Previous studies by Hong et al. linked E7-dependent activation of the ATM pathway with E7-dependent activation of STAT5 (16, 17). However, which domain of E7 is responsible for STAT5 activation has not been established. Furthermore, many previous studies outside the context of HPV infection have linked the dysregulation of the Rb-E2F pathway to DNA damage and ATM activation (13, 37, 43). Given that HPV E7 degrades Rb, dysregulated Rb-E2F signaling could also contribute to E7-mediated ATM activation. Given these studies, in Chapter 2 I hypothesized that the Rb binding domain of HPV E7 was necessary for the upregulation of the ATM pathway during HPV infection.

To test this hypothesis, I established cell lines containing either wild type HPV31 (HFK-31 cells) or an HPV31 genome in which the Rb binding domain of E7 was deleted (HFK-31 ΔLHCYE). In parallel, I established similar cell lines stably expressing either wild type HPV31 E7 (31 E7) or an E7 lacking the Rb binding domain (31 E7-ΔLHCYE). These lines served as a platform on which the rest of the study was built, comparing the levels of proteins or transcripts in these cell lines to the appropriate controls. To examine any phenotypes observed over the entirety of

the HPV life cycle, cells were harvested prior to confluency during log-phase growth (representing the maintenance phase), as well as while differentiating in 1.5% methylcelluose media (representing the productive phase).

I first determined whether the deletion of the Rb binding domain ablates the ability of HPV31 to activate the ATM pathway. As expected, I found that the levels of both phosphorylated (activated) and total ATM and Chk2 were increased in HFK-31 cells relative to uninfected human foreskin keratinocytes (HFKs). In contrast, HFK-31 ΔLHYCE cells completely lost this upregulation. This phenotype was maintained both prior to and after differentiation in methylcellulose. I then examined the levels of Chk1, a protein involved in the activation of the related ATR single stranded break repair pathway. I found that levels of phosphorylated (activated) and total Chk1 were increased in HFK-31 cells but not HFK-31 ΔLHCYE cells across my time course. Together, these data suggest that the Rb binding domain of E7 is necessary to induce elevated levels of ATM, Chk2, and Chk1 in HFK-31 cells. To confirm these results, I examined levels of these proteins in cells expressing E7 alone. As expected, the levels of phosphorylated and total ATM, Chk2, and Chk1 were increased in cells expressing wild type 31 E7 relative to vector control (pLXSN), but not in those expressing 31 E7-ΔLHCYE. Together, these data suggest that the Rb binding domain of HPV31 E7 is necessary to induce increased levels of DDR proteins in HPV infected cells.

Given these data, I determined whether the Rb binding domain of E7 played a role in increasing the levels of other DNA repair factors during HPV infection. Outside of HPV infection, the MRN complex (consisting or MRE11, Rad50, and

NBS1) acts as a sensor for double-stranded DNA breaks (DSBs), activating ATM in

response to DNA damage. In contrast, BRCA1 and Rad51 function downstream of

ATM activation by regulating homologous repair (HR) (10). Previous studies have

established that the levels of these proteins are increased in HPV31 positive and 31

E7 expressing cells, and that the MRN complex, BRCA1, and Rad51 are required for

productive HPV replication (2, 7). As expected, I found that the levels of all three

MRN complex proteins as well as BRCA1 and Rad51 were increased in HFK-31

cells, but not in HFK-31 ΔLHYCE cells, relative to uninfected HFKs across my time

course. Similar results were seen in 31 E7 and 31 E7-ΔLHCYE expressing cells.

Together, these data demonstrate that the Rb binding domain HPV31 E7 is

necessary to induce increased levels of DNA repair factors during HPV infection.

Taken with the fact that Rb-E2F dysregulation can activate DDR outside of

infection (13, 37, 43), these data allowed me to construct a model of ATM pathway

activation during HPV infection. I postulated that HPV E7 increases the levels of

DNA repair factors during HPV infection through the dysregulation of the Rb-E2F

pathway. Thus, deletion of the Rb binding domain of E7 ablated increases in DNA

repair factor levels by preventing activation of E2F dependent transcription.

Preliminary evidence was consistent with this, as both E2F1 and E2F2 levels were

increased in HFK-31 and 31 E7 expressing cells, but not HFK-31 ΔLHYCE or 31 E7-

ΔLHYCE cells (it should be noted that this phenotype is consistent with previous

reports for E2F2) (26). However, when I examined the transcript levels of these DNA

repair factors in HPV31 positive cells, I found no statistically significant changes for

most transcripts. Briefly, only *BRCA1* showed a statistically significant increase in

transcript levels when comparing HFK and HFK-31 cells. When comparing HFK-31 and HFK-31 ΔLHCYE cells, only *BRCA1* and *Rad51* transcripts were significantly reduced. Similar results were obtained in cells expressing E7 alone. Together, these data indicate that while increased transcription plays a role in increasing the levels of a subset of DNA repair factors in HPV31 positive cells, increased transcription is not completely responsible for the increased levels of DNA repair factors during HPV infection.

Based on these data, I hypothesized that HPV31 increases levels of DDR proteins by increasing protein stability. Consistent with this hypothesis, our lab previously published that BRCA1 and Rad51 exhibit increased half-lives in HPV positive cells relative to uninfected HFKs (7). Using cycloheximide, a potent inhibitor of translation, I measured protein degradation over a 12-hour time course in HFK, HFK-31, and HFK-31 ΔLHYCE cells and then cacluated the protein half-lives by linear regression analysis. Examining ATM, Chk2, and Chk1 degradation, I found that the half-lives of ATM and Chk2, but not Chk1, were greatly increased in HFK-31 cells relative to both HFK and HFK-31 ΔLHCYE cells. When the experiment was repeated in cells expressing 31 E7 or 31 E7-ΔLHYCE alone, similar results were obtained. Together, these data suggest that HPV31 E7, through its Rb binding domain, increases the levels of ATM and Chk2 by greatly increasing their stability.

Encouraged by these findings, I next investigated whether proteins in the MRN complex are regulated in a similar manner. I found that MRE11, Rad50, and NBS1 all exhibited increased half-lives in HFK-31 cells relative to HFKs. In HFK-31 ΔLHYCE cells, this phenotype was completely ablated for MRE11. In contrast, an

intermediate level of stabilization was observed for both Rad50 and NBS1 in HFK-31 ΔLHYCE cells when compared with HFKs and wild type HFK-31 cells. When the half-lives of these proteins were examined in cells expressing 31 E7 alone, similar results were obtained. The half-lives of MRE11 and NBS1 were increased in 31 E7 cells when compared with both HFKs and 31 E7-ΔLHYCE cells, which had almost identical half-lives. For Rad50, while the half-life was increased 31 E7 cells compared with HFKs, an intermediate level of stabilization was also observed in 31 E7-ΔLHCYE cells. Together, these data indicate that increased protein stability plays a role in the upregulation of the MRN complex expression levels during HPV infection. These data also suggest that independent of Rb association, HPV retains some ability to stabilize the MRN complex, as the deletion of the E7 Rb binding domain did not completely ablate the phenotype. HPV E1 is a likely candidate, as the expression of E1 alone can also induce DNA damage (41).

Finally, while we previously published that BRCA1 and Rad51 show increased half-lives in HPV31 positive cells, I wanted determine whether the E7 Rb binding domain was necessary for this stabilization (7). I found that both BRCA1 and Rad51 showed increased half-lives in HFK-31 cells compared with HFKs, a phenotype that was lost in HFK-31 ΔLHYCE cells. When cells expressing 31 E7 were compared with HFKs and 31-E7 ΔLHYCE cells, a similar phenotype was observed. Together, these data indicate that BRCA1 and Rad51 have increased half-lives in HPV31 positive cells, and that this phenotype requires the E7 Rb binding domain.

Given these findings I then considered a new model whereby the E7 Rb binding domain is necessary for the upregulation of DNA repair factors during HPV infection, and the dysregulation of E2F dependent transcription cannot completely account for the increased levels of DNA repair factors. Thus, the Rb binding domain of E7 is important for increasing the stability of DDR proteins. A number of hypotheses can be made, though it is difficult to speculate which may explain the observed phenotypes. The simplest possibility is that a single function of E7 causes the upregulation of all of the observed factors, but to date there has not been a "smoking gun" to which this phenotype can be attributed.

An attractive hypothesis is that HPV E7 activates ATM directly, and the subsequent increase in ATM kinase activity is responsible for increasing protein stability. The Discussion section of Chapter 2, describes precedence for ATM regulating protein stability in this manner as ATM is known to phosphorylate the ubiquitin ligase MDM2 which it inactivates to stabilize both p53 and Chk2 (24, 29). Additionally, ATM can stabilize Chk1 and NBS1 through the phosphorylation of ZEB1 and p300, respectively (20, 21, 51). Thus, activation of the ATM kinase may be responsible for the stabilization of the other DNA repair factors during HPV infection. However, as discussed in Chapter 3, inhibition of the ATM kinase exhibits no effect on the levels of DDR proteins in HPV31 positive CIN612 cells, with the sole exception of Rad51. These results suggest that ATM kinase activity is dispensable for the upregulation of DNA repair factors during HPV infection. Thus, E7 must upregulate the stability of DDR proteins independent of E7's activation of the ATM kinase activity.

As discussed in Chapter 1, a large number of proteins require the "Rb binding domain" in order to interact with the HPV E7 protein, perhaps making it more appropriate to refer to the Rb binding domain as the LXCXE motif (see Table 1.1 and reference 39). A likely hypothesis for this observation is that one of the proteins known to bind to E7 through the LXCXE motif is responsible for the broad increase in DNA repair factor stability. One candidate is p300,which as previously mentioned, regulates the stability of NBS1 (5, 20, 21). Because E7 and p300 directly interact, it is possible that E7 induces NBS1 stability independent of ATM activation. Moreover, E7 binds ATM, Rad50, NBS1, and BRCA1 (2, 31, 52). Thus, E7 may directly increase the half-lives of these proteins through direct binding. Consistent with this, E7's interaction with ATM and NBS1 have been shown to require the LXCXE motif (2, 31). Interestingly, E7 binding to ATM is ablated by the inhibition of ATM kinase activity, suggesting that at least in the case of ATM, direct binding cannot explain the increase in protein half-life (31).

Another possibility is that E7 somehow directly interferes with the machinery that regulates turn-over of DNA repair factors. E7 has been shown to inhibit the anaphase promoting complex/cyclosome (APC/C), a ubiquitin ligase complex that has been shown to regulate DDR in addition to its canonical function of regulating mitosis (12, 49). While its exact role is poorly defined, APC/C is activated in response to DNA damage where it modulates the stability of target proteins (12, 44). E7's interaction with the complex may stabilize DDR factors in an undetermined manner.

One important area of future research will be linking STAT5 activation to the Rb binding domain. As mentioned, previous studies by Hong et al. linked the ability of E7 to activate STAT5 to the activation of Tip60, whose acetylation of ATM is required for ATM activation independent of HPV infection (10, 15, 17). However, the mechanism linking the deletion of the E7 Rb binding domain to the activation of both STAT5 and Tip60 has not been elucidated. Knockdown of STAT5 was sufficient to decrease levels of ATM, Chk2, BRCA1, and Rad51; however, whether STAT5 knockdown results in decreased transcript levels or a loss of protein stability has not been investigated. In order to establish a complete mechanism of ATM activation by HPV E7, it will be important to clarify the exact contribution of STAT5.

INDUCTION OF THE G$_2$/M CHECKPONT IN THE STRATEFIED EPITHELIUM

Chapter 3 of this dissertation examined the function of ATM kinase activity in the HPV life cycle by determining the role of ATM in the induction of the G$_2$/M checkpoint in differentiating keratinocytes. Previous studies established that HPV replicates its genome while the host cell is arrested in G$_2$, as evidenced by the cytoplasmic localization of cyclin B and Cdk1 (4, 46). It was found that the levels of proteins regulating the G$_2$/M checkpoint are increased in HPV infected cells as well as cells expressing E7 alone. Importantly, the E7 Rb binding domain was shown to be required for increased levels of cytoplasmic Cdk1 and cyclin B in E7 expressing organotypic raft cultures (4). Given that the ATM mediated DNA damage response is a known inducer of the G$_2$/M checkpoint (10), and that both G$_2$ arrest and ATM activation require the Rb binding domain (4, 23), I hypothesized that ATM activation is necessary for G$_2$/M arrest in HPV infected cells.

To test this hypothesis, I utilized the small molecule inhibitor of ATM kinase activity KU-55933, which was previously shown by our lab to inhibit productive HPV replication and ATM activation during HPV infection (31). Use of this inhibitor allowed me to determine whether the inhibition of the ATM kinase affects the levels of many proteins regulating the G_2/M checkpoint. I found that while levels of cyclin B were unaffected by ATM inhibition both prior to and after differentiation in high calcium media, the total levels of its binding partner Cdk1 were specifically reduced upon differentiation. When the phosphorylation levels of all three regulatory sites in Cdk1 were examined (Thr14 and Tyr 15 are inhibitory, whereas Thr161 is activating) similar decreases were observed. Additionally, cdc25b and cdc25c, phosphatases that serve to activate Cdk1 by dephosphorylating Thr14 and Tyr15, showed similar decreases in expression levels in cells that were differentiated in high calcium media. Finally, Myt1, the kinase responsible for phosphorylating Thr14 of Cdk1 was also decreased upon ATM inhibition upon differentiation. These data suggest that the maintenance of high expression levels of G_2/M proteins upon differentiation requires ATM kinase activity.

While these data demonstrate that ATM inhibition decreases the levels of G_2/M checkpoint proteins in differentiating HPV positive cells, they are insufficient to demonstrate that arrest in G_2 does not still occur. Thus, the next step in this project is to directly determine whether the inhibition of ATM is sufficient to inhibit G_2 arrest in HPV positive cells. An important step in mitotic entry is the re-localization of the cyclin B-Cdk1 complexes from the cytoplasm to the nucleus (38). Activation of the G_2/M checkpoint has been shown to prevent this re-localization. As mentioned,

differentiated HPV positive cells exhibit high levels of cytoplasmic cyclin B and Cdk1. Thus, I am curious whether the inhibition of ATM kinase activity is sufficient to trigger nuclear localization of these proteins. To examine this, I am currently performing immunohistochemistry (IHC) on organotypic raft cultures of HPV31 positive CIN612 cells, shown to faithfully recapitulate the HPV life cycle in the stratified epithelium. Using KU-55933, I will determine whether ATM inhibition is sufficient to induce nuclear localization of both cyclin B and Cdk1 (together known as the MPF), a phenotype that would indicate that HPV positive cells no longer arrest in G_2.

If the above hypothesis is correct, the next step would be to separate any effect that ATM inhibition has on the G_2/M checkpoint from other potential functions of ATM. The ATM kinase has hundreds of downstream targets. Thus, while the inhibition of ATM kinase itself is illuminating, chemical inhibition of ATM is a rather blunt instrument for assessing the role of the ATM pathway in the productive HPV life cycle. Indeed, as detailed in Figure 4.1, there are several functions that ATM may utilize in productive replication, and inhibition of ATM kinase activity blocks all of them simultaneously. Thus, in order to determine whether ATM is necessary for productive replication in part because of ATM activates the G_2/M checkpoint, it is necessary to more thoroughly examine the signaling cascade between ATM activation and inhibition of the MPF. To test this hypothesis, I will first inhibit the activity of the ATM downstream target Chk2, which was also shown by our lab to block amplification of the HPV genome (31). As detailed in Chapter 1, Chk2 is a kinase activated by ATM phosphorylation and plays a major role in activating the G_2/M checkpoint through its inhibition of cdc25 proteins. I hypothesize that inhibition

of Chk2 via the small molecule Chk2 inhibitor II will be sufficient to decrease the expression levels of G_2/M checkpoint proteins and prevent differentiating HPV positive cells from arresting in G_2, as evidenced by nuclear localization of cyclin B in organotypic raft cultures. Next, I would investigate proteins further downstream in the ATM pathway to determine whether Myt1 and Wee1 (the kinases directly responsible for inhibiting Cdk1) activity are also necessary for the induction of the G_2/M checkpoint and productive HPV replication. If either Myt1 or Wee1 are also found to be necessary for the activation of the G_2/M checkpoint and productive HPV replication, then these data would suggest that activation of the G_2/M checkpoint is itself required for productive infection. To demonstrate this further, dominant negative versions of Cdk1 or cdc25c could be expressed. Together, these would demonstrate that the ATM pathway is required for productive HPV replication, at least in part, because it activates the G_2/M checkpoint.

A MODEL OF ATM ACTIVATION AND FUNCTION IN HPV INFECTION

Taken as a whole, my dissertation when combined with the work of others provides evidence for a model of ATM activation by E7 in the replication of the HPV genome (Figure 4.1). During infection, levels of ATM pathway proteins are increased by HPV E7 through its LXCXE motif (also called its Rb binding domain) by broadly upregulating their stability, thus making them available for use in the productive life cycle (23). Many of these factors are then recruited to the viral genome (14). Upon differentiation, the ATM pathway serves several important functions. First, there is evidence that ATM activity is necessary for the recruitment of DNA repair factors to the viral genome through the promotion of certain histone modifications. While

bound, they play a direct role in an HR-dependent replication mechanism, supported by the observation that many of these factors are required for replication (2, 7, 31). Also supporting this hypothesis is the finding that inhibition of MRE11 and Rad51 activity, both of which are required for HR, blocks productive HPV replication (2). Additionally, ATM is known to phosphorylate and activate many of these factors, suggesting the ATM kinase may directly regulate HR to promote the replication of viral DNA (10, 34). Finally, ATM arrests cells in G_2 in the upper layers of the stratified epithelium in order to create an environment conducive to productive replication of the HPV genome (4, 46).

While research regarding this model is ongoing, several key insights from my dissertation can be made. The most enigmatic of these is the observation that DNA repair factors are largely regulated at the level of protein stability, with some showing no statistically significant difference in transcript levels between HPV31 positive and uninfected cells. The observation that ATM activity is dispensable for the upregulation of protein levels further complicates any potential explanation of how the levels of these DNA repair factors are increased. Cleary, there are unrecognized aspects of HPV biology at play, the elucidation of which are required to understand how the ATM pathway is activated. Additionally, connecting ATM activation to G_2/M arrest begins the process of fitting ATM activation into a biological (i.e. needed to arrest the cell cycle) rather than a merely biochemical (i.e. ATM is needed to activate Chk2) framework, allowing the field to see the "bigger picture." This shift will allow the development of new hypotheses that may lead to the development of novel antivirals capable of minimizing HPV related disease.
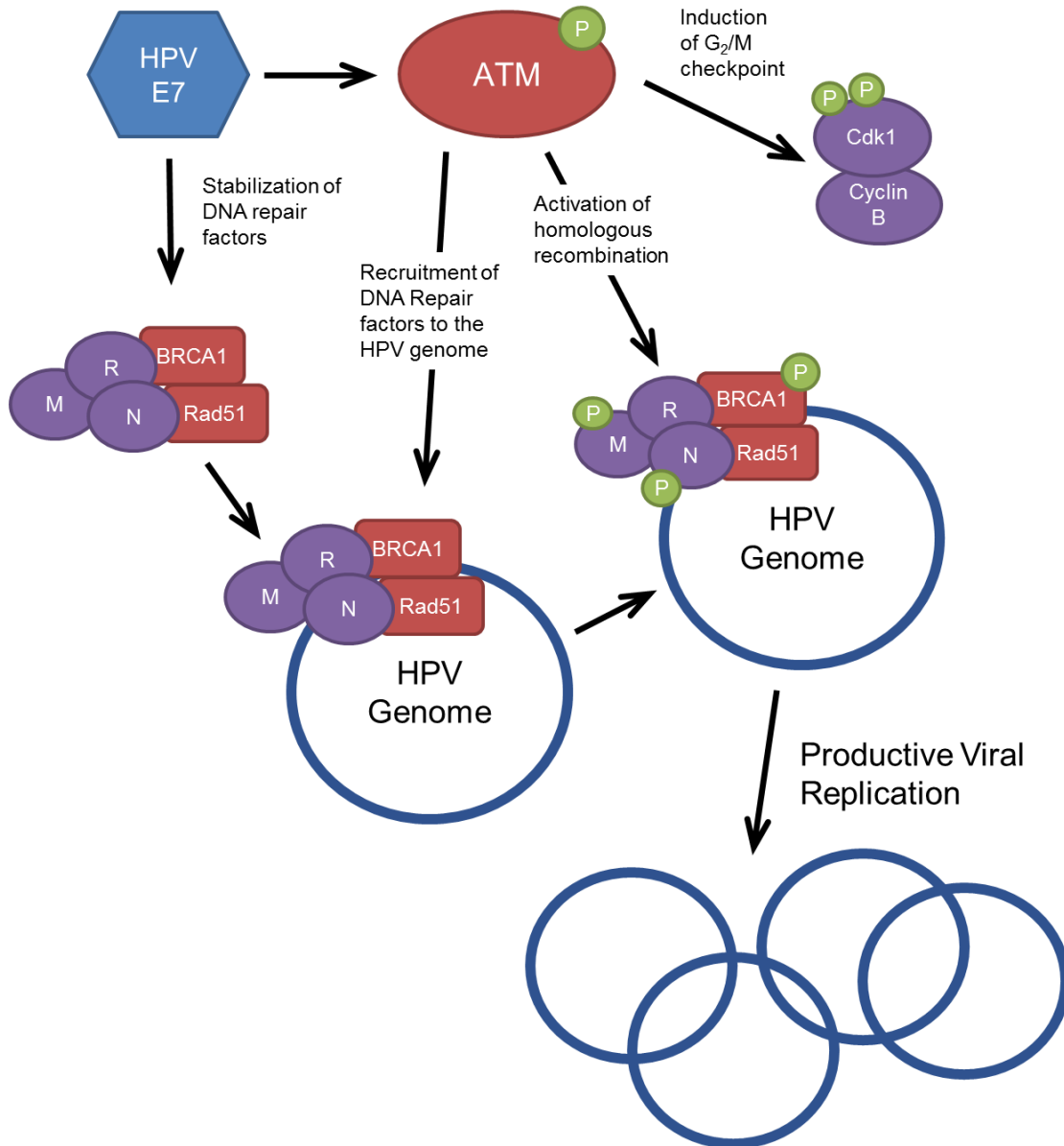
Figure 4.1 HPV utilizes the ATM DDR pathway to replicate the viral genome.

Shown is a hypothetical model of the function of ATM activation during HPV

infection. Briefly, HPV E7 stabilizes DNA repair factors and activate ATM

independently. The ATM protein then facilitates the recruitment of repair factors to

the genome, induces the G_2/M checkpoint, and activates homologous recombination

in order to amplify the viral genome.

DISSERTATION IMPACT: THE POTENTIAL ROLE OF E7-DEPENDENT

UPREGULATION OF DNA REPAIR FACTORS IN ONCOGENESIS

As mentioned above, HPV is the etiological agent of nearly all cervical cancers and has increasingly been linked to other forms of cancer as well (53). Given that HPV E7 is one of the major oncoproteins of HPV, it is curious that HPV E7 increases the levels of ATM pathway proteins, as ATM signaling is generally thought to be tumor suppressive (11, 30). Nevertheless, there are several mechanisms by which E7-dependent upregulation of DDR factor levels may contribute to the development of cancer.

Integration of the HPV genome into host chromosomes is a hallmark of HPV related oncogenesis (36, 47). Increased levels of HPV integration are associated with the progression from low to high grade precancerous cervical intraepithelial neoplasias (CINs), and can be used as a marker of disease progression (3, 18, 19). Integration of the HPV genome often ablates the regulation of early gene expression by E2, leading to increased expression of E6 and E7, increased cellular proliferation, and is believed to promote oncogenesis (18, 22). Additionally, integration itself can modulate the expression of nearby cellular genes which hypothetically could promote oncogenesis (35, 42). To date, no "hot spots" for integration have been identified, although so-called fragile sites (areas of chromosomes that are particularly susceptible to damage) have been suggested by some studies (45). Despite the frequency at which HPV integration has been observed in tumors and its association with the progression of precancerous CINs, the mechanism by which the HPV genome is integrated into the host chromosome has not been determined.

Microhomology (MH) refers to short sections within two DNA sequences that are identical, with the majority of these sequences lacking significant similarity. A recent study by Hu et al. performed a genome-wide analysis of integration sites on 135 samples containing HPV DNA (26 CINs, 104 cervical carcinomas, and 5 established cell lines). They demonstrated that chromosomal regions immediately flanking sites of HPV integration have significantly enriched amounts of MH with the ends of integrated HPV genomes (18). This observation suggests that integration of the HPV genome may occur through an MH-based mechanism. There are three known mechanisms of MH-based DNA repair and replication that may be involved in HPV integration. These include microhomology-mediated end joining (MMEJ), fork stalling and template switching (FoSTeS), and microhomology-mediated break-induced replication (MMBIR) (32). Of these three pathways, the molecular details of MMEJ are best understood. Like HR, MMEJ occurs most frequently in the S and G_2 phases of the cell cycle and requires DNA resection by the MRN complex and CtIP (25, 28, 48). However, unlike the relatively accurate HR pathway, MMEJ is error-prone and commonly results in the deletion, duplication, or rearrangement of small sections of DNA (32). How the cell decides between the activation HR or MMEJ is poorly understood, but MMEJ is believed to exist as a "back up" repair pathway that is induced when DNA repair by HR is unsuccessful. Rather than relying on significant homology between sister chromatids, as in HR, MMEJ joins free DNA ends with limited amounts of homology (5-25 bp, i.e. MH), resulting in less accurate repair (28). In contrast, FoSTeS and MMBIR involve the collapse of replication forks followed by the invasion of the replicating DNA strand into DNA sequences sharing

MH sites. After strand invasion, replication restarts using the invaded regions as a templates. Strand invasion in FoSTeS and MMBIR can occur in multiple successive rounds, resulting in an often complex pattern of duplications and deletions depending on the number of strand invasion events and on the location(s) of the MH site (50).

As discussed throughout this dissertation, many proteins involved in HR are increased in HPV positive and E7 expressing cells (2, 7, 31). Interestingly, a subset of the factors upregulated by E7 also function in MMEJ (25, 48). However, other factors, such as BRCA1, function to promote HR and inhibit MMEJ (32). Given that HPV E7 expression broadly increases ATM pathway proteins and that the proteins regulating all three MH-based pathways are poorly described, it is difficult to determine the role that increased levels of DDR factors may play in HPV genome integration. Nevertheless, several promising hypothetical models can be considered. In the case of an MMEJ-based integration mechanism, the first step is the spontaneous occurrence of DNA damage in an HPV infected basal keratinocyte. E7 may increase the frequency of such events by increasing oxidative and replicative stress (6, 8, 27). HR begins with the successful resection of the DSB. However, when HR fails, the MMEJ pathway is activated to rescue DNA repair. Because of MH shared between the damaged section of the host chromosome and the HPV genome, MMEJ then mistakenly incorporates existing HPV genomes into the host chromosome resulting in integration. HPV E7's upregulation of DDR proteins may facilitate this process.

Alternatively, HPV may be integrated by a FoSTeS or MMBIR based mechanism. In this model, free ssDNA resulting from collapsed replication forks invades HPV genomes in a process mediated by shared MH. Replication then resumes on the invading ssDNA strand using the HPV genome as a template. Thus, the HPV genome is integrated into host chromosomes through MH-mediated replication rather than direct incorporation of existing HPV genomes. As mentioned, E7-dependent induction of replication stress may increase the frequency by which this occurs (6). Additionally, E7-dependent upregulation of DDR factors and their recruitment to viral genomes may facilitate replication by this mechanism. In conclusion, E7-mediated manipulation of DDR protein expression may contribute to oncogenesis in part by increasing the likelihood of HPV integration.

HPV E7-dependent upregulation of DDR factors may function in cancer progression independent of integration of the HPV genome as well. Indeed, ATM signaling has been reported to be upregulated in some forms of cancer, including melanoma, prostate, and pancreatic cancers (11). In such cases, ATM is thought to promote cell survival under stress in situations where apoptosis and cell cycle dysregulation have already been subverted (11). Given that apoptosis is efficiently subverted by E6-dependent degradation of p53 and that E7 subverts cell cycle control in addition to activating ATM, it is possible that increased levels of DDR proteins in HPV infected cells serve to promote cancer progression by ensuring the survival of infected cells. Consistent with this idea, inhibition of ATM in cell lines derived from cervical carcinomas decreases cell survival (i.e. radioresistance) in response to irradiation, suggesting that ATM pathway activation promotes survival of

cervical cancer cells (40). Additional research is needed to investigate the validity of this model, as the degree to which DDR proteins serve to promote cell survival in different grades of precancerous lesions and HPV infections (compared with cervical carcinoma lines) has not been examined.

A final model for the role for HPV E7-dependent upregulation of DDR factors in oncogenesis derives from the current ambiguity regarding the extent to which DNA repair is active in HPV infected cells. While research from our lab and others has firmly established that DDR protein levels are increased in HPV and E7 expressing cells, it is less clear as to whether DNA repair is itself activated. At least one study suggested that DNA repair has delayed kinetics in response to high risk HPV E7 expression, despite increased levels of the HR protein Rad51 (33). Thus, in an alternative model, DNA repair by HR may be *less* efficient in HPV infected cells resulting in an increased rate of oncogenic mutations despite having increased levels of DNA repair factors. If true, then this model begs the question as to why high levels of DNA repair factors do not facilitate DNA repair. One possibility is that HPV upregulates DNA repair proteins required for replicating its genome and then sequesters the factors to replication foci (see Figure 4.1), thereby preventing them from participating in the repair of host DNA.

The differentiation between the accuracy of these model(s) of HPV E7-induced oncogenesis will provide insights into the development of cervical and other forms of HPV related cancers. Given the long temporal lag between initial HPV infection and cancer development, elucidation of the exact details of HPV induced oncogenesis may allow for the development of interventions to prevent HPV related

disease. Given that cervical cancer still results in the death of over 270,000 women

annually worldwide and that current vaccines are not therapeutic (53), such

interventions are vitally important for public health.

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