MANIPULATION OF THE HOST CELL DNA DAMAGE PATHWAYS BY HUMAN PAPILLOMAVIRUS

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ABSTRACT

Daniel Carl Anacker: Manipulation of the Host Cell DNA Damage Pathways by Human Papillomavirus
(Under the direction of Cary Moody)

Human papilloma virus (HPV) is thought to be the most common sexually transmitted viral infection in the United States. It poses a major public health risk since persistent infection with certain types of HPV is a major risk factor for several cancers. HPV is highly adapted for immune evasion and follows a strictly regimented life cycle in order to evade immune detection. The HPV life cycle is closely tied to host cell differentiation with late viral events, such as structural gene expression and viral genome amplification taking place in the differentiating upper layers of the epithelia, removed from immune detection. The virus accomplishes this through a complex system of host cell manipulation, and tight control of its own gene expression and genome replication

This dissertation addresses how the virus, with its very limited coding capacity, has managed to commandeer the many host factors required to successfully replicate the viral genome. I specifically investigated how the virus, especially the viral oncogenes E6 and E7 interface with the ATM and ATR dependent DNA damage response (DDR), in order to create an atmosphere conducive to productive viral replication in a differentiating keratinocyte. First, we expanded on previous work that indicated the ATM DDR response was constitutively

activated in HPV positive cells and necessary for successful productive viral genome replication. We determined that Nbs1, a protein involved in the ATM DDR pathway, known to be recruited to sites of HPV replication, was required for productive viral genome replication. However, we found that Nbs1 plays a role in viral genome amplification outside of its ability to activate ATM. Our evidence suggests that Nbs1 may recruit other proteins, involved in homologous repair (HR), that may be needed for productive viral replication. We next investigated how the virus may be activating the ATR DDR in order to provide other factors necessary for viral genome synthesis. Previous research has shown that the ATR DDR is activated in HPV positive cells and that levels of the ribonucleotide reductase (RNR) small subunit M2 (RRM2) are upregulated. In this dissertation we show that levels of deoxyribonucleotide triphosphates (dNTPs) are elevated in HPV positive cells, both prior to and post differentiation. We have found that RRM2 levels in these cells are upregulated in an ATR/Chk1/E2F1 dependent manner and that RRM2 is necessary for viral genome replication, especially upon differentiation.

This dissertation is dedicated to my parents who taught me the value of hard work and always encouraged my scientific curiosity.

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LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATMIN	ATM interacting
ATR	Ataxia telangiectasia and Rad3 related
CDC	Cell division cycle
C/EBP	CCAAT-enhancer-binding proteins
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CtIP	CtBP interacting protein
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
dNTP	Deoxyribonucleotide triphosphates
dNDP	Deoxyribonucleotide diphosphates
DSB	Double strand DNA break
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
HEK	Human embryonic kidney
HFK	Human foreskin keratinocyte
HPV	Human papillomavirus
HR	Homologous repair
HSPG	Heparin sulfate proteoglycans
HSV	Herpes simplex virus

HU	Hydroxyurea
IP	Immunoprecipitation
KSHV	Kaposi’s sarcoma-associated herpesvirus
LCR	Long control region
MRN	Mre11-Nbs1-Rad50
mRNA	Messenger RNA
NBS	Nijmegen breakage syndrome
NHEJ	Non-homologous end joining
ORF	Open reading frame
PEI	Polyethylenimine
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative Real time polymerase chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
RRM1	Ribonucleotide reductase subunit M1
RRM2	Ribonucleotide reductase subunit M2
SCF	Skp, Cullin, F-box containing
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SIRT1	Sirtuin 1
SMC	Structural maintenance of chromosomes
SV40	Simian Virus 40
TOPBP1	Topoisomerase (DNA) II binding protein 1
UTR	Untranslated region

VSV	Vesicular stomatitis virus
YY1	Yin Yang 1

CHAPTER 1: INTRODUCTION

OVERVIEW

Human Papilloma Virus (HPV) is a double stranded non-enveloped DNA virus that infects epithelial cells known as keratinocytes. Specifically, alpha-HPVs target the mucosal epithelia of the anogenital tract and oropharynx. The range of HPVs can be further broken down into viral subtype with viral genome sequence and antibody recognition differing significantly between viral subtypes. Broadly they can be divided into two groups, high and low risk, based on their association with cancer. While low risk subtypes such as HPV 6 and 11 typically cause benign warts, the high risk subtypes, including HPV 16, 18, 31, and 45 are known to cause cervical, penile, anal, and head and neck cancers. Cervical cancer alone is the second leading cause of cancer deaths among women with 490,000 cases and 270,000 deaths worldwide each year (1). HPV is thought to be the most common sexually transmitted viral infection, with an overall prevalence of 26% in US females aged 14 to 59 and a peak prevalence of over 44% in women ages 20-24 (2). Since among HPV subtypes, high risk HPVs pose the largest known threat to public health, my dissertation research specifically focused on these viruses.

INTRODUCTION

The HPV genome exists as a circular episome of 7.5 to 8kb in length depending on the specific viral subtype. The genome typically encodes eight proteins; early proteins E1, E2, E4, E5, E6, and E7 and the late proteins L1 and L2.

The early proteins are non-structural and are involved in a variety of functions ranging from viral genome replication to cell cycle control. E1 is the virally encoded helicase that also recruits host DNA replication factors to the viral origin of replication (3). E2 is the viral transcription factor that also cooperates with E1 to facilitate the initiation of viral genome amplification (4-7). E6 and E7 are viral oncoproteins that perform a wide variety of functions ranging from anti-apoptotic functions to cell cycle manipulations (7-9). L1 and L2 are the two structural proteins that form the viral capsid. The functions of proteins E4 and E5 have not been completely described. E4 is also commonly referred to as E1^E4 as it is expressed from a spliced mRNA consisting of the first 5 amino acids of the E1 ORF followed by the entire E4 ORF (10). E5 is thought to interact with cellular EGFR receptors and play an undetermined role in viral genome amplification (11). The HPV life cycle, which is tightly tied to host cell differentiation can be described in 3 main stages; establishment, maintenance, and amplification, each of which involves viral genome replication. However, since the HPV genome does not encode any polymerases or many other proteins necessary for viral genome replication, the virus must rely on host DNA synthesis machinery in order to replicate the viral genome.

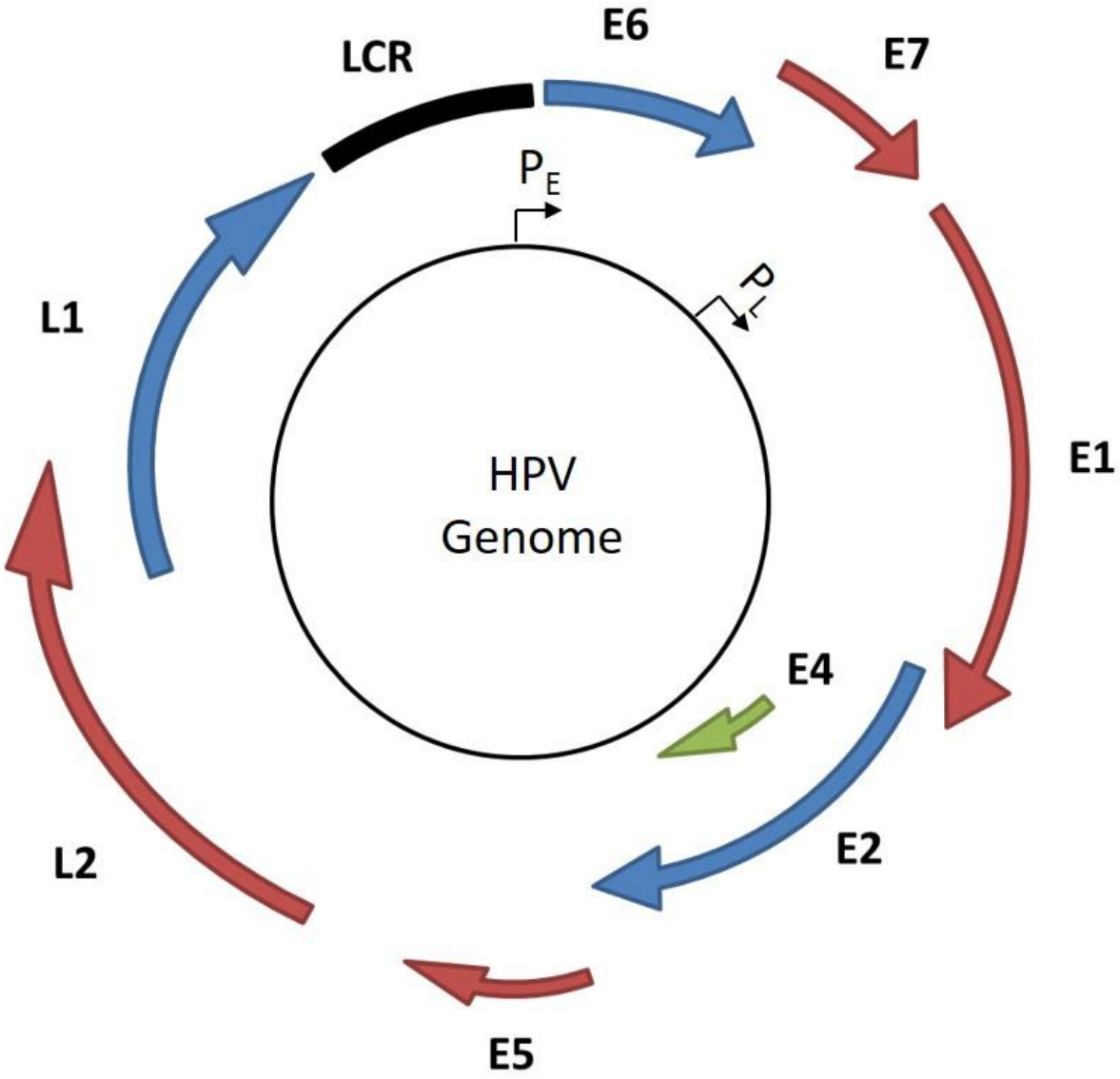

Figure 1.1 HPV viral genome organization. Open reading frames are indicated with arrows and the long control region (LCR) is indicated by a black square. The early and late promoters are also indicated (P_E and P_L respectively).

THE DIFFERENTIATION DEPENDENT VIRAL LIFE CYCLE

Initial infection: As previously mentioned HPVs, are known to target mucosal epithelia, specifically the mucosal epithelia of the anogenital tract and

oropharynx (12). While this specific tropism has been well described, the actual process of viral attachment and entry are not completely understood. Infection is known to require small epithelial abrasions known as microwounds (8). These micro wounds are tiny tears in the epithelia that allow virions to access the basement membrane. Once bound to the basement membrane the virion is able to come into contact with the basal layer of keratinocytes. Once in contact with the basal keratinocyte, L1 capsid protein on the virion surface is thought to attach to heparin sulfate proteoglycans (HSPGs) on the cell surface (13). After the initial attachment event there is a conformational change in the capsid structure and the L2 protein is cleaved by host cell furin (14). At this point it is currently thought that the virion binds an unknown secondary cellular receptor which mediates viral entry into the cell. The mechanism by which the virus enters the cell is still unclear. A variety of entry pathways have been reported, leading to the suggestion that the viral entry mechanism may be viral subtype dependent (15).

Establishment Phase: Upon Host cell entry the virus un-coats and unloads its genome into endosomes (12). Viral genomes are then released from the endosomes into the cytoplasm, this process has been shown to be dependent on the same L2 furin cleavage event that has been shown to play a role in viral attachment (16). After the viral genomes have entered the cytoplasm, L2 is also involved in transferring the cytoplasmic genomes to the host cell nucleus (5). Once in the host nucleus, in order for persistent infection to be established early proteins must be expressed. These proteins include the viral replication proteins E1 and E2, along with the oncoproteins E6 and E7. Expression of the transcripts for these

proteins is mediated by the early promoter which is located directly upstream of the E6 coding region (17). The activity of the early promoter is controlled through the binding of positive and negative factors to the upstream long control region (LCR) (18-20). At this point the viral protein E2 is very important as it is the major viral transcription regulator, and regulation of viral transcription is thought to be at least partially responsible for controlling viral copy number in undifferentiated cells (21). Viral genome replication also requires E1 protein, the viral helicase (22). Acting together in complex, E1 and E2 are able to bind the viral origin of replication with high affinity (22, 23). However once the complex is bound to the viral origin of replication, which is adjacent to the early promoter, E2 is released from the complex before DNA replication begins (22). E1 is then responsible for recruiting the cellular factors required for viral genome replication and unwinding the viral DNA (23).

Maintenance phase: Once stable infection has been established the virus enters what is referred to as a maintenance phase infection. During this phase, viral genomes are stably maintained at a level of 50 to 100 copies per cell and the early proteins E1, E2, E6, and E7 continue to be expressed from the early promoter (24). Recent evidence suggests that during the maintenance phase the viral genome is replicated along with the host cell genome and is distributed equally to daughter cells upon cell division (22). E2 has been identified as a necessary factor to ensure equal separation of HPV genomes to daughter cells by tethering viral genomes to host chromosomes (25). The viral oncoproteins E6 and E7 are also necessary for the stable maintenance of viral genomes as episomes during the maintenance stage of infection (26). During maintenance phase, E6 is also active in binding and

degrading p53 (8). This activity prevents host cell apoptosis and inhibits cell cycle checkpoints. E7 expression during the maintenance phase is important because of its ability to promote destruction of retinoblastoma protein (Rb). The degradation of Rb deregulates the cell cycle and helps to push the cell towards a proliferative state by releasing E2F transcription factors which activate transcription of S phase genes important for DNA replication (8).

Productive phase: The virus remains in the maintenance phase while the host cell remains among the basal layer of keratinocytes. When an infected basal cell divides, one of the daughter cells remains in the actively dividing basal layer while the other begins to move upward, away from the basal layer and begins to differentiate. This activation of host cell differentiation activates the productive phase of the viral life cycle (8).

While a great deal about the link between host cell differentiation and triggering of the productive phase of HPV infection remains to be explained, some differentiation related triggers have been discovered. The viral protein E2 is important in controlling viral gene expression; the LCR region upstream of the early promoter contains multiple E2 binding sites (20, 27, 28). While a small amount of E2 binding has been shown to activate transcription from the early promoter, large amounts of E2 binding have been shown to down regulate the use of this promoter (29). Upon differentiation the cellular transcription factors C/EBP1α and C/EBPβ are more strongly expressed (30). These transcription factors have been shown to act synergistically with E2 enhancing their activity. Another way in which differentiation triggers the activation of late viral gene expression is by modulating factors that

affect the late viral promoter, located downstream of the early promoter within the E7 coding region (17, 31). It has been suggested that upon differentiation the binding affinity of transcriptional repressors YY1 and Cux1 for the late promoter is decreased possibly allowing greater transcription (11). Finally, there is evidence that upon differentiation, a change in the balance of expression between transcription factor SP1 and its antagonist SP3 and the increase in transcription factor C/EBPβ activate the late promoter (11, 32, 33).

Aside from E2, other non-structural proteins have roles to play upon differentiation and induction of late viral events. E6 and E7 have been shown by Moody and Laimins to trigger caspase activation upon differentiation resulting in the cleavage of E1 (34). In the absence of this cleavage, a defect in viral genome replication was observed, suggesting caspase mediated cleavage activity was necessary for successful viral amplification (34). Upon differentiation E7 continues to bind and degrade Rb family proteins, releasing E2F transcription factors. This release of E2F factors pushes the differentiating cell back into the cell cycle and again triggers important S-phase genes necessary for DNA synthesis (19).

DNA DAMAGE REPAIR

Reliable maintenance of a stable and accurate genome is an essential function of every cell. In order to ensure faithful replication and maintenance of genomes several mechanisms exist to detect and repair damage to DNA. Depending on the type of damage that occurs, the cell has a variety of repair pathways at its disposal. As a group these pathways are known as the DNA damage response (DDR). Two major regulators of the DDR are the Ataxia telangiectasia

mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases which, along with the DNA-dependent protein kinase (DNA-PK) are members of the PI 3-Kinase related kinase family of kinases (35). Double strand DNA breaks (DSBs) can be repaired by either the high fidelity, homologous repair (HR) response regulated by ATM or the more error prone non-homologous end joining (NHEJ) response (35). Conversely damage resulting from replication stress and stalled DNA replication forks is handled by a pathway regulated through ATR (36, 37). HPV infection exploits both the ATM and ATR DDR pathways in order to successfully replicate its genome (38). It is thought that through these pathways, HPV maintains and recruits host cell factors necessary for viral genome replication, especially upon differentiation. It is therefore important to describe these two major DDR pathways and how they are exploited by HPV infection.

ATM DNA damage repair pathway: The ATM DDR pathway is typically activated in response to DNA DSBs and primarily repairs DNA through HR. In this case, the DNA DSB is typically detected by the MRN complex which is composed of Mre11, Rad50, and Nbs1 (35). Rad50 is a member of the structural maintenance of chromosomes (SMC) family of proteins and interacts with the broken ends of DNA in the DSB (35). Mre11 facilitates MRN complex formation through binding both Rad50 and Nbs1. It also has endonuclease and exonuclease activities important for preparing broken DNA ends for HR (39, 40). Nbs1, besides interacting with Mre11 also directly binds ATM (41, 42). Upon recognition of the DSB the MRN recruits ATM to the site of damage where it binds to Nbs1 and is activated via auto phosphorylation (43-45). However, ATM can be activated outside of this classical

pathway. The competing ATM interacting (ATMIN) protein has been shown to activate ATM in a MRN in a Nbs1 independent manner in response to hypotonic stress and inhibition of DNA synthesis by drugs like Hydroxyurea (HU) (46). Additionally, the DNA repair protein 53BP1 has been shown to be an activator of ATM, especially when levels of MRN are low (47, 48). Activated ATM then coordinates repair of the DSB, phosphorylating a plethora of downstream targets including proteins involved in HR such as Nbs1, Brca1, and CtIP (49). Aside from recruiting ATM to the site of the DSB, the MRN complex stabilizes the break while MRN complex protein Mre11, along with ATM targets CtIP and Brca1 mediate resection of the broken ends to allow for recruitment of Rad51 mediated by Brca2 (50) reviewed in (35). The newly recruited Rad51 replaces phosphorylated RPA protein on the resected strands of broken DNA leading to strand invasion of the homologous template, formation of the D-loop, and ultimate repair through HR (51). Portions of this process are also dependent on ATR and its downstream effector Chk1. ATR, along with ATM, have been shown to phosphorylate RPA (48). Additionally, Chk1 has been shown to phosphorylate Rad51 (52). Both of these events are important for the formation of Rad51 filaments on the resected DNA ends and eventual stand invasion and repair of the DSB via HR (53).

Other important targets of ATM involved in DSB repair include histone H2AX, Chk2, and p53 (54-56). Phosphorylated or γH2AX is an important marker of sites of DNA damage. γH2AX foci radiate outward from the site of the DSB and serve as a docking site for many other DNA damage repair proteins (57). These γH2AX foci can extend for up to two megabases beyond the site of damage and assist in the

recruitment of many repair factors including 53BP1, Nbs1, Brca1 and Rad51 (35) (3, 58, 59). This allows for the amplification of the repair response through the recruitment of additional factors to the area of damage. Phosphorylation of Chk2 results in arrest in G2 phase through inactivation of CDC25 and its downstream effector CDK1 (60). This cell cycle arrest allows time for DNA repair before continuation of the cell cycle. Activation of p53 also leads to cell cycle arrest and in the case of unrepaired damage, cell death through apoptosis (56).

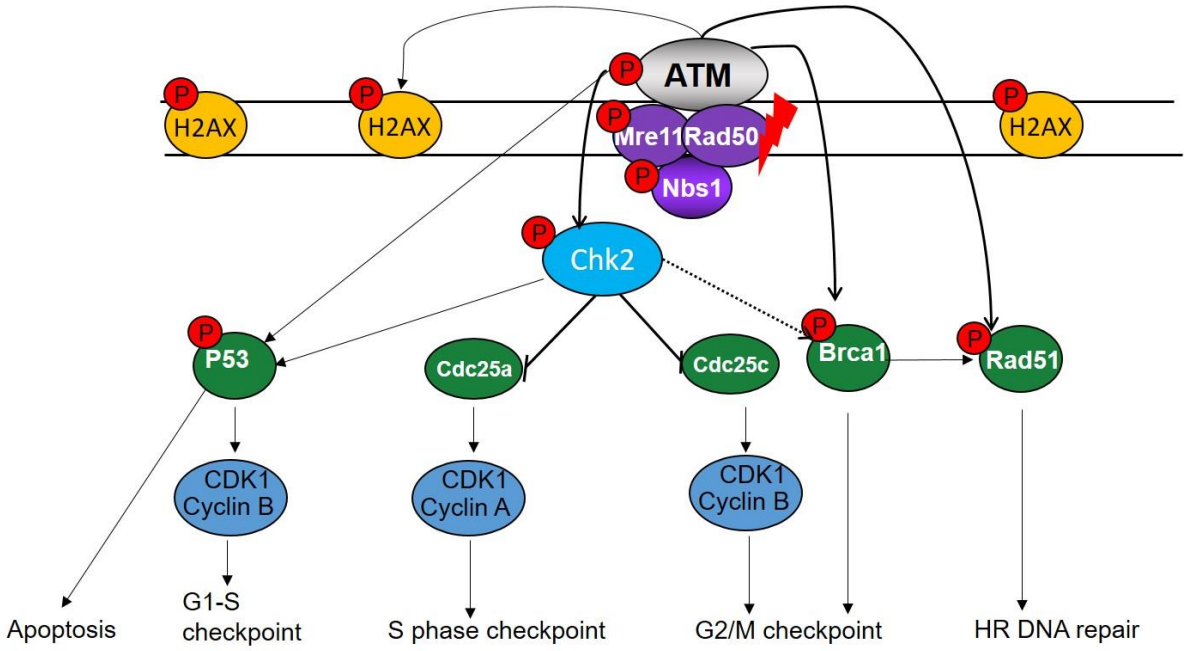

Figure 1.2 The ATM dependent DNA damage signaling. A DNA double strand break (indicated by the lightning bolt) is detected by the MRN complex which then recruits ATM. ATM then phosphorylates several downstream effectors.

ATR DNA damage pathway: While ATR and its downstream effectors Chk1 and Chk2 have been shown to be active in the ATM DDR pathway as outlined

above, ATR is also known to be the central coordinator of DDR relating to replication stress and replication fork stalling (36, 37). In the case of replication stress large regions of ssDNA coated with RPA are formed due to the decoupling of the helicase and polymerase (35, 61). Polymerization of RPA1 on this ssDNA recruits ATR and its partner protein ATRIP through a direct interaction between RPA1 and ATRIP (61). In this way ATR is localized to the replication fork. At the same time the RPA-ssDNA complex also recruits the RAD17-RFC2-5 clamp loader, which in turn loads the 9-1-1 complex (RAD9-HUS1-RAD1) bound to the ATR activating TOPBP1 (36, 62-65) (reviewed in (35)). This cascade of events leads to the activation of ATR at the stressed replication fork. Activated ATR is then able to signal through its downstream effectors Chk1 and Chk2. As discussed above Chk2 activation results in cell cycle delay. Activation of Chk1 by replication stress has recently been shown to result in high-level expression of genes related to DNA repair and nucleotide synthesis including the ribonucleotide reductase (RNR) small subunit M2 (RRM2) (66). Other studies have shown that ATR/Chk1 activation promotes RRM2 accumulation by stabilizing E2F1 which transcriptionally activates RRM2 expression. This ATR/Chk1/E2F1 mediated increase in RRM2 is essential for providing dNTPs to prevent DNA damage and cell death (66-68).

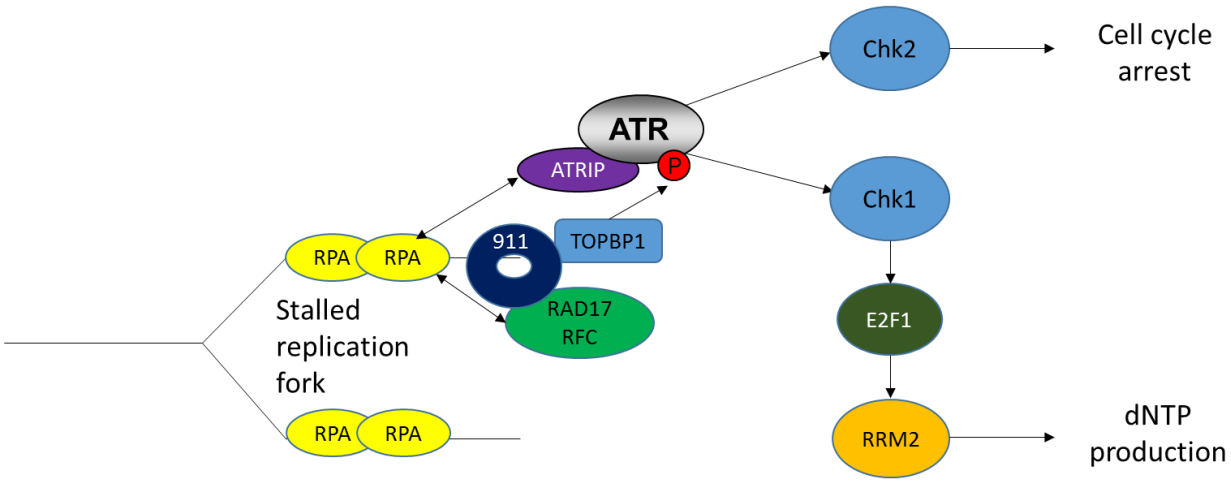

Figure 1.3 The ATR DNA damage response. ATR is recruited to sites of replication stress and stalled replication forks. ATR signaling through Chk1 can result in increased RRM2 expression and nucleotide production to allow for DNA repair.

The ribonucleotide reductase enzyme: In proliferating cells, two RRM2 subunits together with two of the larger RRM1 subunits form the RNR tetramer (69). This enzyme responsible for the reduction of ribonucleotide diphosphates to deoxyribonucleotide diphosphates (dNDPs) which are subsequently phosphorylated by the nucleoside diphosphate kinase to produce the balanced pools of dNTPs necessary for DNA synthesis. Outside of an actively dividing cell RRM2 can be replaced in the RNR enzyme by the p53 reactive subunit p53R2. This happens most often in G0/G1 phase when dNTP usage is typically limited and related to mitochondrial DNA replication and repair (69, 70). A properly regulated and steady supply of dNTPs is essential for successful DNA synthesis so activity of the RNR enzyme is tightly controlled through the cell cycle. While RRM1 has a long half-life and levels stay steady throughout the cell cycle, RRM2 is relatively short lived and is transcriptionally regulated in an E2F1 dependent manner, with levels peaking during

S-phase (71-73). Outside of S-phase RRM2, is actively degraded through proteasome mediated degradation, facilitated by the anaphase promoting complex/Cdh1 in G1 (74) and by $SCF^{cyclinF}$ in G2 (75). The increase in RRM2 levels during S-phase coincides with an increase in RNR activity at the same time, suggesting that RRM2 is the rate limiting component of the RNR enzyme (76, 77). Confirming this, a loss of RRM2 results in decreased abundance of dNTPs available for DNA synthesis (78, 79). Appropriate RNR activity is important for maintenance of genomic integrity and cell viability. An increase or imbalance in dNTP levels can lead to mutations (80) while insufficient dNTP pools can impair DNA replication and repair (69).

HPV MAINIPULATION OF THE DNA DAMAGE RESPONSE

The ATM DDR: Many viruses are known to interact with and manipulate the DNA damage response. Replication-coupled recombination is thought to play a role in the life cycle of several herpes viruses where activated HR proteins have been observed and the MRN complex is even thought to play a role in maintenance of latent EBV episomes (81-83). In HPV infected cells, manipulation of the ATM DDR and the recruitment of many ATM DDR and HR proteins to viral genomes is known to take place throughout the viral lifecycle (84, 85). Importantly, manipulation of the ATM regulated HR pathway has been shown to be necessary for productive viral amplification upon host cell differentiation (84-86). HPV productive replication is known to require the activity of several proteins involved in HR. One of the first ATM DDR proteins discovered to play a role in the HPV lifecycle was the master regulator ATM itself (84). Moody and Laimins first observed activated ATM as well as

downstream effectors Chk2, and Brca1 in HPV positive cells. In infected cells HPV E7 forms a complex with phosphorylated ATM through its LXCXE Rb binding domain. In addition, E7 is able to activate Chk2 phosphorylation, possibly through its interaction with ATM. This activation of ATM and Chk2 in HPV positive cells was sustained upon differentiation, where the downstream substrate Nbs1 was also found to be phosphorylated. While not necessary for viral genome maintenance in undifferentiated cells, ATM and Chk2 activation were both necessary for successful productive viral replication upon differentiation. Interestingly, Chk2 activation upon differentiation in these cells is necessary for the caspase 7 activation described above, suggesting one possible pathway connecting Chk2 activation with replication (84). Furthermore, as previously described, activation of Chk2 by ATM leads to a G2/M cell cycle arrest through the cytoplasmic sequestration of CDC25C which prevents activation of CDK1. HPV productive replication is believed to occur in a G2/M arrested state (87). Therefore, the requirement for ATM activation, specifically for productive replication suggests that HPV may be exploiting this pathway in order to hold the host cell in a state conducive for productive viral replication.

Another downstream effector of ATM thought to play a role in the HPV lifecycle is the histone variant H2AX. Phosphorylated or γH2AX is found associated with HPV DNA throughout the viral life cycle and these γH2AX foci expand upon productive viral replication (58). This association suggest that γH2AX may play an important role in recruiting HR factors to sites of HPV genome replication. Supporting this hypothesis, the deacetylase SIRT1 has been shown to bind at DNA damage foci and recruit Nbs1 and 53BP1 in a γH2AX mediated manner (88).

Upregulation of SIRT1 has been observed in HPV positive cells; this upregulation has been shown to be important for the recruitment of Nbs1 and Rad51 to HPV genomes and necessary for productive viral replication (89, 90).

Some viruses, like adenovirus, actively antagonize the MRN complex and catalyze its destruction, preventing Nbs1 phosphorylation (91). In contrast, HPV infected cells maintain high levels of Mre11, Rad50, and Nbs1 both pre and post differentiation (85). This maintenance of MRN components and the phosphorylation of Nbs1 observed in differentiating infected cells suggest that Nbs1 may also be important in regulation viral replication. Further suggesting the importance of the MRN, recently our lab has shown that the HR factors Rad51 and Brca1 are necessary for productive viral replication (86). As explained above, the MRN complex plays an important role in the recruitment of Rad51 and Brca1 to DNA double strand breaks where they are necessary for the completion of HR.

In addition to HPV E7 interacting with ATM and activating a DNA damage response HPV E1 is also capable of activating an ATM dependent DNA damage response (3, 59, 92) reviewed in (93). Both the DNA binding and helicase activities of E1 have been shown necessary in triggering this response and when both E1 and E2 are expressed in a cell they form foci on the host chromatin (3, 59). These results suggest that E1 may be triggering a DNA damage response in these cells by melting regions of the cellular chromatin around the foci (92). Similar to the foci observed by Gillespie et al. in differentiating HPV positive cells these E1-E2 foci recruit members of the ATM DDR pathway including pATM, γH2AX, pChk2, Nbs1, and Mre11 (3, 58). In addition to the observation of the recruitment of these factors, labeled nucleotides

were incorporated at these foci, indicating DNA synthesis and/or repair activity (3, 94).

The ATR DDR: While manipulation of the ATM DDR pathway by HPV has been studied for some time, less is known about HPV engagement of the ATR DDR pathway. However, recent studies have presented data suggesting that HPV manipulates and requires the ATR DDR throughout its lifecycle (3, 84, 92, 95). It is known that expression of the HPV oncogenes E6 and E7 cause replication stress (96, 97). This is most likely due to the cell being pushed into S-phase as described above, which may trigger DNA replication in the absence of the required materials. Previous studies have shown that ATR and Chk1 are constitutively activated in HPV positive cells, further suggesting the presence of ongoing replication stress in these infected cells (3, 84, 92, 95). Outside the context of a full infection expression of both E7 (95) and the viral helicase E1 have been shown to be sufficient to trigger the activation of ATR and Chk1 (3, 92). This is important because inhibition of Chk1 has been shown to decrease the stability of viral genomes in HPV infected cells prior to differentiation (51), and even more recently Chk1 activity has been shown to be necessary for viral genome amplification upon differentiation. As stated above ATR mediated Chk1 activation has been shown to lead to RRM2 accumulation. Taken together these observations suggest that activation of the ATR-Chk1 pathway may be another way in which HPV infection increases RRM2 levels in order to insure a steady supply of dNTPs for viral DNA synthesis.

RATIONALE FOR DISSERTATION

Due to its small coding capacity and lifecycle being so tightly linked to host cell differentiation, HPV has evolved many ways of manipulating the host cell in order to recruit and regulate host factors necessary to replicate the viral genome. Recently, there has been a great deal of research into how HPV manipulates the host cell by targeting the ATM and ATR DDR pathways. In this dissertation I attempt to further our understanding of HPV manipulation of these pathways. Specific proteins involved in these pathways targeted by the virus are identified along with the viral proteins responsible for the manipulation.

In the second chapter I focus on the ATM DDR and determine that Nbs1, a member of the MRN complex is necessary for productive HPV replication. However, despite the fact that Nbs1 is known to play an important role in ATM activation and ATM activity is also known to be necessary for productive viral replication, we found that depletion of Nbs1 had a minimal effect on ATM activation. Instead, our data suggests that Nbs1 is important for productive replication because it recruits HR factors such as Mre11 and Rad51 to sites of HPV replication. In fact, we show that Mre11 nuclease activity is also necessary for productive viral genome replication.

In the third chapter I examine the interaction of the ATR DDR and HPV, a considerably less explored topic. We propose a pathway in which RRM2 levels are upregulated though the release of E2F1 factors due to Rb degradation and stabilization of E2F1 activity through the activation of ATR and Chk1. We believe that the observed increase in RRM2 resulted in the increased dNTP pools that we measured in HPV positive cells. This lead us to the hypothesis that the virus was

specifically targeting RRM2 expression through this pathway, in order to provide dNTPs for viral genome replication.

Hopefully further research into HPV manipulation of these two important cellular pathways will lead to a better understanding of how we can control HPV replication and spread. Additionally, we may identify strategies to treat the persistent HPV infections that are known to be associated with many cancers.

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CHAPTER 2: PRODUCTIVE REPLICATION OF HUMAN PAPILOMAVIRUS 31 REQUIRES THE DNA REPAIR FACTOR NBS1[1]

OVERVIEW

Activation of the ATM (Ataxia telangiectasia-mutated kinase)-dependent DNA damage response (DDR) is necessary for productive replication of Human Papillomavirus 31 (HPV31). We previously found that DNA repair and homologous recombination (HR) factors localize to sites of HPV replication, suggesting ATM activity is required to recruit factors to viral genomes that can productively replicate viral DNA in a recombination-dependent manner. The Mre11-Rad50-Nbs1 (MRN) complex is an essential component of the DDR that is necessary for ATM-mediated HR repair, and localizes to HPV DNA foci. In this study, we demonstrate that the HPV E7 protein is sufficient to increase levels of the MRN complex, and also interacts with MRN components. We have found that Nbs1 depletion blocks productive viral replication, and results in decreased localization of Mre11, Rad50, and the principal HR factor Rad51 to HPV DNA foci upon differentiation. Nbs1 contributes to the DDR by acting as an upstream activator of ATM in response to double strand DNA breaks (DSB), and as a downstream effector of ATM activity in

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the intra-S phase checkpoint. We have found that phosphorylation of ATM and its downstream target Chk2, as well as SMC1 (structural maintenance of chromosome 1) is maintained upon Nbs1 knockdown in differentiating cells. Given that ATM and Chk2 are required for productive replication, our results suggest that Nbs1 contributes to viral replication outside of its role as an ATM activator, potentially through ensuring localization of DNA repair factors to viral genomes that are necessary for efficient productive replication.

INTRODUCTION

Human papillomavirus are small double stranded DNA viruses that exhibit a strict tropism for epithelial cells (1). A subset of HPV types (termed high-risk) are the causative agents of cervical cancer, and are also associated with other genital malignancies, as well as an increasing number of head and neck cancers (2). The life cycle of HPV is dependent upon the differentiation of its host cell, the keratinocyte. There are three phases of viral replication that characterize the viral life cycle (3). Upon infection of basal keratinocytes, the virus transiently amplifies to 50-100 episomal copies per cell. In undifferentiated cells, the virus is maintained at a low copy number by replicating once per cell cycle along with cellular DNA (4). In contrast, upon keratinocyte differentiation, the productive phase of the viral life cycle is activated, resulting in late gene expression, viral genome amplification to thousands of copies per cell, virion assembly and release (1). Viral genome amplification is thought to occur through multiple rounds of replication following cellular DNA synthesis in cells arrested in an S- or G2-like environment (5-8), with

some evidence indicating this occurs through a switch to rolling circle replication (9). Although it is well established that the viral E7 protein promotes S phase re-entry of differentiating cells to provide cellular factors necessary for productive replication (10), the mechanisms that regulate the switch to viral genome amplification in differentiating cells are not well understood.

Over the past several years, it has become evident that DNA and RNA viruses facilitate replication by targeting the DNA damage response (11). Previously, we showed that high-risk HPV31 induces constitutive activation of an ATM-dependent DNA damage response throughout the viral life cycle (12). ATM is a member of the phosphatidylinositol 3-kinase-like kinases (PIK) family of kinases, which along with ATR (Ataxia telangiectasia and Rad3-related protein) and DNA-PK, respond to certain types of DNA damage (13). ATM and DNA-PK are typically activated in response to double strand DNA breaks (DSB), while ATR is activated in response to single stranded DNA breaks, as well as replication stress. Our previous studies demonstrated that HPV31 requires ATM kinase activity for productive replication upon differentiation, but not for episomal maintenance in undifferentiated cells (12). In HPV31 positive cells, the ATM response was characterized by phosphorylation of downstream targets, including Chk2, Nbs1 and Brca1 (12). Similarly to inhibition of ATM, Chk2 inhibition also blocked productive replication, indicating an important role for ATM signaling specifically during the differentiation-dependent phase of the viral life cycle. How HPV activates ATM is currently unclear, though we have found that E7 expression alone is sufficient to induce activation of ATM targets (12), possibly through the induction of replication stress and DNA

damage (14, 15). Recent studies by Hong and Laimins demonstrated that E7-induced STAT5 activation is necessary for ATM activation, possibly through PPARγ expression (16). We, as well as others, have shown that expression of viral helicase E1 can also stimulate ATM activation (8, 17). Why HPV requires ATM activity for productive replication, as well as which ATM effectors contribute to viral DNA synthesis is not well understood.

In more recent studies, we demonstrated that multiple components of the ATM DNA damage response pathway localize to sites of HPV replication, including γH2AX, Chk2, 53BP1 and components of the MRN complex (Mre11, Rad50, Nbs1) (18). In addition, we found that Rad51 and Brca1, two proteins necessary for the repair of DSBs through homologous recombination (HR) (19), are increased in expression in HPV positive cells and localize to viral DNA foci. The localization of cellular replication factors, PCNA and RPA32, to HPV DNA foci indicated these were sites of viral DNA synthesis. In addition, we observed that RPA32 is phosphorylated at sites of HPV replication, which is thought to redirect RPA's function from DNA replication to repair synthesis, and has been linked to DSB resection (20-22). This suggests that HPV may utilize ATM signaling to recruit DNA repair machinery to viral genomes to promote replication through DNA repair. In support of this, we found that γH2AX is bound to viral chromatin throughout the viral life cycle, with binding increasing upon productive replication (18).

Increasing evidence suggests a role for recombination in HPV replication (23). Indeed, the localization of Rad51 and Brca1 to sites of HPV DNA synthesis suggests that productive replication may result in structures that require HR for

processing. HR is a high-fidelity repair mechanism that requires ATM activity and functions to rejoin DSBs and restart broken replication forks (19, 24). Replication-coupled recombination is thought to play role in the life cycle of several viruses, including Simian Virus 40 (SV40) (25), Herpes Simplex Virus type 1 (HSV-1) (26-28), Epstein Barr virus (EBV) (29, 30), and Kaposi's Sarcoma Associated Herpesvirus (KSHV) (31). The MRN complex is also essential to homology-directed repair (32). The MRN complex serves as a sensor of DNA damage that also controls the DDR through activation of ATM (33-35). ATM is recruited to sites of DSBs by directly binding Nbs1, where ATM activates the DNA damage checkpoint and regulates DNA repair by phosphorylating specific substrates (36-38). In addition to facilitating ATM activation and recruitment to DSBs, Nbs1 also acts downstream as an effector of ATM activity, and initiates HR with Mre11, a nuclease involved in resection of DNA ends (22, 39). The importance of Nbs1 is facilitating DNA repair is evident in patients with Nijmegen breakage syndrome (NBS), a disorder due to hypomorphic mutations in the Nbs1 gene, which is characterized by cellular radiosensitivity, cell cycle abnormalities and a defective response to DNA damage (40-42). Several viruses have been shown to relocalize and/or degrade components of the MRN complex to facilitate viral replication (11). In addition, the SV40 Large T antigen (43), as well as the HSV protein UL12 (44) has been shown to interact with MRN components. Our previous studies demonstrated that HPV positive cells exhibit high levels of MRN components throughout the viral life cycle (12), which may be important in HPV's ability to activate ATM, and therefore contribute to efficient of viral replication upon differentiation.

In this study, we investigate whether Nbs1 and maintenance of the MRN complex has an impact on the ability of HPV to efficiently replicate. We report here that HPV31 and HPV16 E7 bind to the MRN components Nbs1 and Rad50, but not Mre11. However, formation of the MRN complex is not disrupted, and rather is increased compared to uninfected keratinocytes. We have found that Nbs1 is required productive viral replication, but not episomal maintenance. Depletion of Nbs1 results in a loss of Mre11, Rad50 as well as Rad51 from sites of viral replication upon differentiation, suggesting that productive replication may occur through a mechanism dependent on recombination. Although phosphorylation of ATM and Chk2 are decreased in the absence of Nbs1, relatively high levels remain during the productive phase of the viral life cycle. Importantly, our results indicate that Nbs1 does not contribute to productive viral replication solely as an upstream regulator of ATM activity, but rather has functions downstream as well, with Nbs1 potentially acting as an effector of ATM activity and/or ensuring efficient viral DNA synthesis through a recombination-dependent mechanism.

MATERIALS AND METHODS

Cell culture. Human foreskin keratinocytes (HFKs) were collected from neonatal foreskin tissue as described previously (45) and were maintained in Dermalife keratinocyte growth media (KGM; Lifeline Cell Technology). The human cervical carcinoma cell line C33A was grown in DMEM supplemented with 10% bovine growth serum (BGS) (Life Technologies). CIN612 9E cells, which are derived from a CIN1 biopsy and stably maintain HPV31 genomes, were grown in in E-media supplemented with 5ng/mL mouse epidermal growth factor (BD Biosciences) and

co-cultured with mitomycin C treated J2 3T3 fibroblasts, as described previously (45). The NBS-ILB1 fibroblast cell line was a generous gift from K. Cerosaletti, and was described previously (46). NBS-ILB1 cells were maintained in DMEM with sodium pyruvate supplemented with 10% BGS. Generation and maintenance of cells stably expressing pLXSN or pLXSN-HPV31 E7, pLXSN-HPV16 E7 through retroviral transduction has been previously described (47). When necessary, J2 feeders were removed from HPV positive cells by incubating with Versene (PBS containing 1mM EDTA). U20S and 293T cells were grown in DMEM supplemented with 10% BGS.

Plasmids and Chemicals. The pBR322min-HPV31 plasmid has been described previously (48). The HA-tagged HPV31 E7 proteins were previously described (49) and are as follows: HA-E7 ΔLHCYE contains an in-frame deletion of the Rb binding domain, and the HA-E7 L67R construct contains a point mutation in the HDAC binding site, converting a leucine to an arginine. The TAP-tagged HPV16 E7 construct was generously provided by J. Bodily, and was described previously (50). The retroviral plasmids pLXIN, pLXIN-Nbs1, and those expressing Nbs1 truncation mutants (Nbs1 652 and Nbs1 ΔATM) were kind gifts from P. Concannon, and were described previously (51, 52). The pLXIN-Nbs1 FR5 deletion construct was obtained from K. Cerosaletti and was described previously (52). The Mre11 binding mutant was also obtained from K. Cerosaletti, and was generated by site-directed mutagenesis (QuikChange, Agilent Technologies) based on the identification of the Mre11 binding domain by You et al (53), resulting in a NFKK684-

687AAAA conversion. KU-55933 was obtained from Calbiochem, and MIRIN was obtained from TOCRIS.

Generation of HPV 31 positive HFKs. HFKs stably maintaining HPV31 episomes were created as previously described (45). Briefly, HPV31 genomes were excised from the pBR322 plasmid using HindIII (New England Biolabs) and religated using T4 DNA ligase (Life Technologies). Primary HFKs were transfected with 1ug of the ligated genomes and 1ug pSV2-Neo using FuGene 6 according to manufactures instructions (Promega). Stable cell lines were generated through neomycin selection (Sigma-Aldrich). After selection was complete, pooled populations were expanded for further analysis.

Induction of Keratinocyte Differentiation. For differentiation, 1.5% methylcellulose was used as described previously (54). Cells were harvested at T0 (undifferentiated), as well as 24 and 48 hours post-suspension. High calcium medium was also used to induce differentiation as previously described (7). Cells were harvested at T0, as well as 48, 72 or 96hr post-exposure to high calcium. For both methylcellulose and calcium, at each time point DNA was harvested from one half of the cells, and protein was harvested from the other half. For every experiment, viral genome amplification was measured by Southern blot analysis to ensure activation of the productive phase of the viral life cycle.

Generation of Lentivirus. Lentivirus was produced as previously described (55). Plasmids encoding a Nbs1 shRNA (TRCN0000010393) or a scramble non-target control shRNA in the pKLO background were obtained from Open Biosystems (Pittsburg, PA). Each of these plasmids (5ug) was co-transfected with 1.6ug vesicular stomatitis virus G plasmid DNA, and 3.37ug Gag-Pol-Tet-Rev plasmid DNA into 293T cells using polyethyleneimine (PEI) (VWR). Supernatants containing lentivirus were harvested three days post-transfection, filter sterilized, and stored at -80°C until use. CIN612 9E and HFK-31 cells were transduced with 5ml viral supernatant consisting of Scramble or Nbs1 shRNA lentivirus particles in the presence of 4.8ug/mL hexadimethrine bromide (Polybrene) (Sigma-Aldrich) for three days, followed by selection in puromycin to generate stable cell lines. Knockdown of Nbs1 was confirmed for each experiment by Western blot analysis.

Nuclear/Cytoplasmic Fractionation. Fractionation was carried out according to the methods of Schreiber et al (56). Briefly, after washing in cold PBS, cells were resuspended in 400uL Schreiber buffer A (10mM HEPES, 0.4M NaCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF) and swollen on ice for 15 minutes. The cells were then lysed by the addition of 25ul 10% NP-40 and vortexing for 10 seconds. The nuclei were subsequently pelleted by centrifugation at 4˚C. The supernatant containing the cytoplasmic extract was removed and the nuclei were lysed by addition of 50ul Schreiber buffer C (20mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF) and shaking for 15 minutes at 4˚C. The soluble nuclear fraction was separated by centrifugation for 5 min at 4˚C. Purity

of nuclear and cytoplasmic fractions was determined by Western blot analysis using antibodies to Lamin A/C (Genetex) and Tubulin (Sigma-Aldrich), respectively.

Western blot analysis/Immunoprecipitation. For Western blotting, whole cell lysates were harvested in RIPA lysis buffer supplemented with Complete Mini and PhosSTOP tablets (Roche). Total protein levels were determined via Bio-Rad protein assay. Western blot analysis was performed as described previously (49). Equal protein amounts were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). For immunoprecipitations, cells were harvested in 1X cell lysis buffer (Cell Signaling), as described previously (12). The following primary antibodies were used: anti-mouse HA, anti-rabbit HA, anti-goat Nbs1, anti-mouse involucrin, anti-mouse Ku70, anti-mouse CDC25C, anti-mouse Cyclin A, anti-rabbit Cyclin B, anti-rabbit RPA32, anti-mouse GAPDH (Santa Cruz); anti-mouse Cyclin E (Pharmingen); anti-mouse HPV16 E7 (Life Technologies); anti-rabbit Nbs1, phospho-Nbs1 S343 (Novus Biologicals); anti-rabbit phospho-ATM S1981 and anti-mouse CDK2, anti-rabbit PCNA (Abcam); anti-rabbit phospho-Chk2 Thr68 and Chk2, anti-rabbit CDK1, and anti-rabbit SMC1 (Cell Signaling Technologies); anti-rabbit ATM, anti-rabbit phospho-SMC1 Ser966 (Bethyl Laboratories); anti-mouse Lamin A/C, anti-mouse Mre11 and anti-rabbit Rad50 (Genetex); anti-mouse Tubulin (Sigma-Aldrich). Secondary antibodies used were: HRP conjugated anti-goat (Santa Cruz), HRP conjugated anti-rabbit (Cell Signaling Technologies), and HRP conjugated anti-mouse (GE Life Sciences).

Southern Blot analysis. DNA isolation and Southern blotting were performed as previously described (54). Briefly, cells were harvested in buffer containing 400mM NaCl, 10mM Tris pH 7.5 and 10mM EDTA. Cells were lysed by the addition of 30uL 20% SDS and subsequently treated with 15ul of 10mg/mL proteinase K overnight at 37˚C. DNA was then extracted by phenol chloroform and precipitated using sodium acetate and ethanol. Resultant DNAs were digested with BamHI (which does not cut the HPV31 genome) or HindIII (which cuts the HPV31 genome once). DNAs were resolved on a 0.8% agarose gel and transferred to a positively charged nylon membrane (Genescreen plus; Perkin Elmer). Hybridization was performed using ^{32}P-labeled HPV31 genome as a probe.

Immunofluorescence (IF) and Fluorescence in situ hybridization (FISH). CIN612 9E cells stably expressing the scramble or Nbs1 shRNA were grown on coverslips and harvested at T0 or 72 hours following differentiation in high calcium. At the indicated times, the cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes, then permeabilized with 1% Triton-X 100 in phosphate buffered saline (PBS) for 10 minutes, followed by three washes with PBS. Cover slips were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes and incubated with primary antibodies in 3% BSA-PBS overnight at 4°C in a humidified chamber. Cover slips were washed three times with PBS and incubated with secondary antibodies for one hour followed by three washes with PBS. Cover slips were cross-linked with

cold methanol:acetic acid (3:1) at -20°C for 10 minutes followed by fixation with 4% PFA for 10 minutes at room temperature. Cover slips were then analyzed for HPV DNA by FISH using Tyramide-enhanced fluorescence (Invitrogen) as previously described(12, 57). The coverslips were mounted using Vectashield containing DAPI to counterstain the cellular DNA. Images were captured using Zeiss CLSM 710 spectral confocal laser scanning microscope. For IF, the following antibodies were used: mouse monoclonal anti-Mre11 (1:200; GeneTex), mouse monoclonal anti-Rad50 (1:200; GeneTex), and rabbit polyclonal Rad51 (1:200; Santa Cruz). For the secondary antibody, Alexa Fluor® 568 Goat Anti-Mouse was used (Life Technologies). IF/FISH was carried out for each repair factor on three independent experiments. The number of foci positive for both HPV DNA and each repair factor was quantified, with 25 to 40 FISH-positive cells being counted for each experiment.

Real-time PCR. RNA was isolated from HFKs, as well as HFKs stably expressing pLXSN-HPV 31 or -HPV16 E7 using RNA STAT 60 (Tel-Test), followed by treatment with DNase (Promega) according to the manufacturer instructions. RNA was reverse transcribed using the iScript reverse transcription kit (Biorad). 50 nanograms of cDNA was analyzed in triplicate reactions using qPCR with 375nM primers and iTaq Universal SYBR Green Supermix (Bio-Rad) in a total reaction volume of 20ul. Reactions were carried out in an ABI 7500 thermal cycler with a thermal profile of 3 min at 95°C, 40 cycles of 95°C for 15 seconds, then 30 seconds at 60°C, followed by a melting curve to ensure proper annealing. The results were analyzed using version 2.0.5 of the ABI 7500 software application. The following

gene specific primer sequences were used: Mre11 (Forward 5'-GCCTTCCCGAAATGTCACTA -3'; Reverse 5'- TTCAAAATCAACCCCTTTCG -3'), Rad50 (Forward 5'- GGAAGAGCAGTTGTCCAGTTACG -3'; Reverse 5'-GAGTAAACTGCTGTGGCTCCAG -3'), Nbs1 (Forward 5'-CACCTCCAAAGACAACTGCGGA -3'; Reverse 5'-TCTGTCAGGACGGCAGGAAAGA -3'), GAPDH (Forward 3'-CTGTTGCTGTAGCCAAATTCGT -5'; Reverse 3'- ACCCACTCCACCTTTGAC -5'). Relative transcript amounts were calculated using the ΔΔCT method using GAPDH as a reference gene.

RESULTS

Expression of high-risk HPV E7 increases levels of repair factors, including the MRN complex. Previously we demonstrated that HPV31 E7 is sufficient to induce phosphorylation of the ATM target Chk2 (12). To determine if this observation extends to HPV16 E7, we examined Chk2 phosphorylation in human foreskin keratinocytes (HFK) stably expressing pLXSN-HPV16 E7 or vector control. As shown in Figure 2.1A, we found the expression of HPV16 E7, similarly to HPV31 E7 (Figure 2.1B), is sufficient to induce Chk2, as well as Nbs1 phosphorylation, and also significantly increase total levels of Chk2 (Figure 2.1C, D). Our previous studies also revealed that the MRN components Mre11, Rad50 and Nbs1 are expressed at increased levels in HPV positive cells compared to uninfected keratinocytes (12). To determine if E7 could be responsible for increased levels of the MRN components in infected cells, we examined the levels of Mre11, Rad50 and

Nbs1 in keratinocytes stably expressing HPV16 E7 or HPV31 E7. As shown in Figure 2.1, expression of both HPV16 E7 and HPV31 E7 resulted in a significant increase in protein levels of Mre11, Rad50 and Nbs1, however the repair proteins Ku70 and SMC1 were not affected (Figure 2.1 A-D). To determine if the increase in MRN components was regulated transcriptionally, we performed quantitative real-time PCR on RNA extracted from HFKs stably expressing HPV31 E7 or vector control. As shown in Figure 2.1E, although expression of HPV 31E7 resulted in increased transcript levels of Mre11 and Nbs1, only Mre11 was significantly changed. In contrast, Rad50 was significantly decreased at the transcript level in HPV31 E7 cells compared to the vector control. Since Mre11 stabilizes Rad50 and Nbs1 through binding (58, 59), it is possible that the E7-mediated increase in Mre11 transcript and protein levels is sufficient to maintain elevated levels of Rad50 and Nbs1. Overall, these results suggest that in addition to activation of ATM signaling, E7 may be necessary to provide essential DNA repair factors for viral replication.

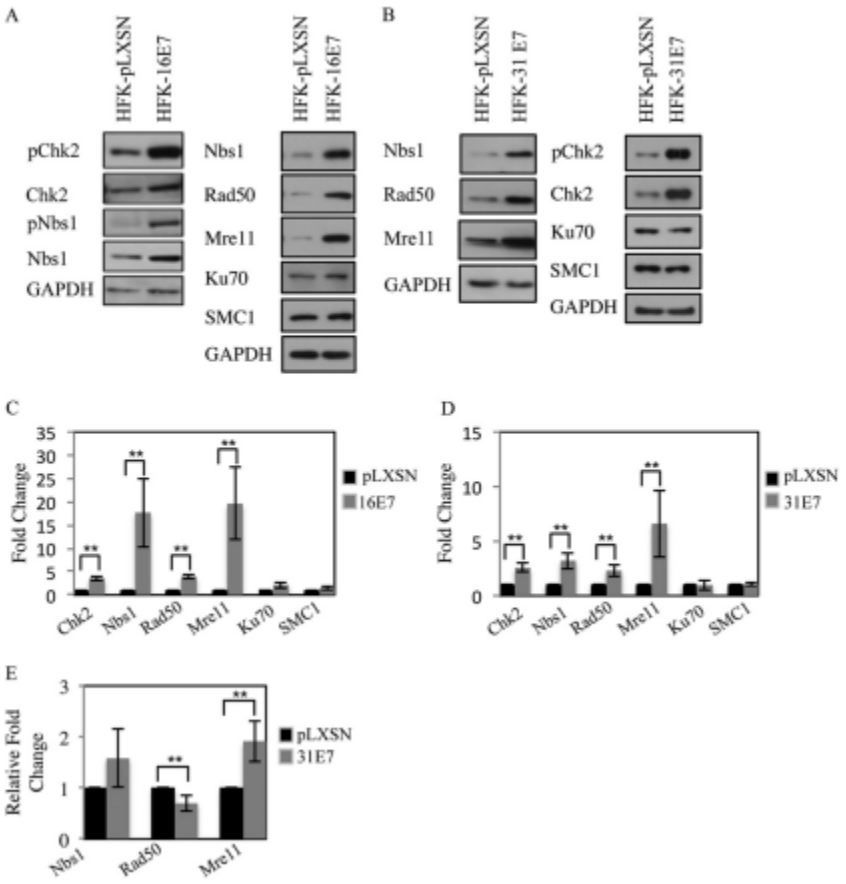

Figure 2.1. Expression of HPV E7 increases levels of proteins associated with detection and repair of DNA damage. (A) Whole cell extracts were harvested from HFKs stably expressing pLXSN vector control or pLXSN-HPV16 E7. Immunoblotting was performed using antibodies to phosphorylated Chk2 (Thr68) (pChk2), total Chk2, phosphorylated Nbs1 S343 (pNbs1), total Nbs1, Rad50, Ku70 and SMC1. (B) Lysates harvested from HFK-pLXSN or HFK-pLXSN HPV31 E7 cells were analyzed by Western blot analysis using antibodies to pChk2, Chk2, Nbs1, Rad50, Mre11, Ku70 and SMC1. GAPDH served as a loading control. (C, D) Bar graphs demonstrate the average expression level of target proteins, normalized to GAPDH, in at least four independent Western blot analyses, including data from Panel A and B. Densitometry was performed using ImageJ software. The statistical analysis was

assayed by 2-tailed t-test. Data=mean +/- standard error. ** indicates p-value less than 0.05. (E) Quantitative real-time (qRT) PCR of gene expression analysis in HFK-pLXSN and HFK-pLXSN-31E7 lines. Expression levels are shown relative to HFK-pLXSN cells and were calculated using GAPDH serving as a reference gene. Shown is the relative fold change in gene expression over three independent experiments. The statistical analysis was assayed by 2-tailed t-test. Data= mean +/- standard error. ** indicates p-value less than 0.05.

E7 binds to the MRN components Nbs1 and Rad50. In previous studies, we found that HPV31 E7 binds preferentially to the phosphorylated form of ATM (12), potentially due to the exposure of the E7 binding site upon dissociation of inactive ATM dimers to active monomers. Since Nbs1 binds ATM for recruitment to DSBs, we wanted to determine if E7 also binds Nbs1. For this, we immunoprecipitated HA tagged HPV31 E7 from transiently transfected U20S cells, and performed Western blotting for endogenous Nbs1. As shown in Figure 2.2A, Nbs1 co-immunoprecipitates with HPV31 E7, and we found similar results when endogenous Nbs1 was immunoprecipitated, followed by Western blot analysis for HPV16 E7 (Figure 2.2B) or HPV31 E7 (Figure 2.2D, 2E). The E7/Nbs1 interaction does not appear to occur nonspecifically through DNA, as immunoprecipitation in the presence of ethidium bromide did not affect the ability of E7 to bind Nbs1 (data not shown).

In order to map the Nbs1 interaction domain on HPV31 E7, we used previously characterized mutants that block either the binding of Rb (ΔLHCYE) or histone deacetylases (HDACs) (L67R) (Figure 2.2C) (49). As shown in Figure 2.2D, both the E7 Rb binding mutant and the HDAC binding mutant were deficient in immunoprecipitating Nbs1. Since we previously found ATM also binds E7 through these two domains (12), we wanted to next determine if E7 interacts with Nbs1 indirectly through its binding to ATM. For this, we treated cells with the small molecule inhibitor of ATM, KU-55933, which inhibits ATM phosphorylation and ablates the ability of E7 to bind ATM (Figure 2.2E) (12). However, we found that Nbs1 was still able to immunoprecipitate E7, even when the E7/ATM interaction was disrupted (Figure 2.2E), indicating that E7 binds Nbs1 independently of ATM.

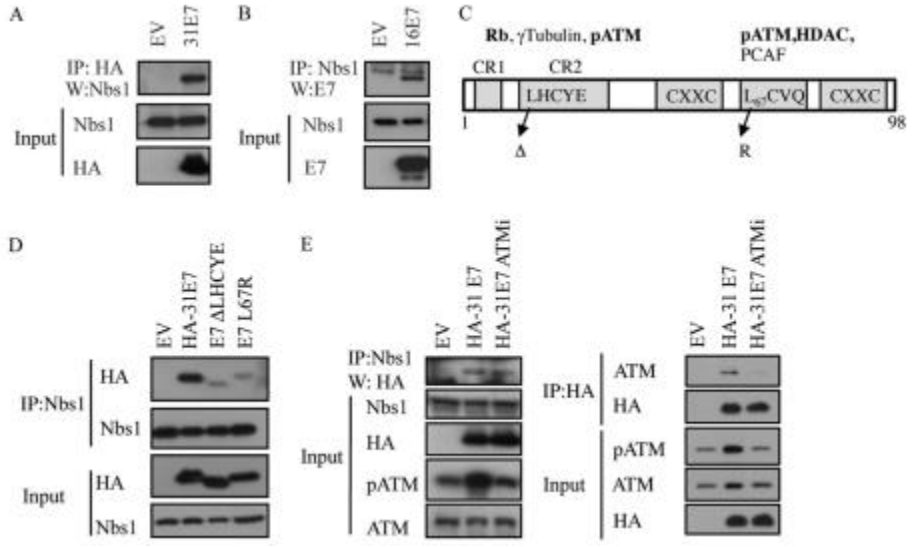

Figure 2.2. HPV E7 interacts with Nbs1 independently of ATM. (A) Whole cell lysates of U20S cells transiently transfected with HA-HPV31 E7 or empty vector (EV) were immunoprecipitated with antibodies to HA or Nbs1, followed by immunoblotting with an antibody to Nbs1 or HA. (B) Whole cell lysates of U20S cells

transiently transfected with TAP-HPV16 E7 or empty vector (EV) were immunoprecipitated using an anti-Nbs1 antibody, followed by immunoblotting with an HPV16 E7 antibody. Inputs were analyzed using antibodies to (A) Nbs1 and HA, and (B) Nbs1 and HPV16E7. (C) Structure of E7 and mutations examined in this study. Indicated are cellular targets that have been shown to interact with the Rb (LHCYE) and HDAC (L67) binding domains(97). (D) Whole cell lysates of U20S cells transiently transfected with EV alone, HA-HPV31 E7, HA-31 E7 ΔLHCYE, or HA-31 E7 L67R were immunoprecipitated using an anti-Nbs1 antibody and subsequently immunoblotted using an anti-HA or anti-Nbs1 antibody. Input lysates were analyzed by Western blot analysis using antibodies to HA and Nbs1. (E) Whole cell lysates of U20S cells expressing EV alone, or HA-HPV31 E7 in the presence or absence of 10uM the ATM inhibitor KU-55933 were immunoprecipitated using an anti-Nbs1 antibody and immunoblotted using an anti-HA antibody (left panel). Input lysates were immunoblotted with antibodies to Nbs1, HA, as well as phosphorylated ATM (Ser1981) and total ATM to demonstrate ATM inhibition. Lysates were also subjected to immunoprecipitation with an antibody to HA, followed by immunoblotting to ATM and HA. Inputs were analyzed using an antibody to HA, as well as phosphorylated and total ATM to demonstrate ATM inhibition. Ab=antibody. IP=immunoprecipitation. W=immunoblotting. ATMi=KU-55933. All results are representative of observations of three or more independent experiments.

To map the HPV31 E7 interaction site on Nbs1, we utilized a series of previously characterized Nbs1 deletion mutants (Figure 2.3A) (51, 52). For these

studies we used the NBS-ILB1 fibroblast cell line that is hypomorphic for Nbs1 and produces undetectable levels of a 70-kDa C-terminal polypeptide (46, 60). NBS-ILB1 cells were retrovirally transduced with wild-type (WT) Nbs1, a mutant that lacks the C-terminus (Nbs1 652), a mutant that lacks the N-terminus (FR5) or a mutant that lacks the ATM binding site (ΔATM), and stable cell lines were generated through neomycin selection. Immunoprecipitation of Nbs1 from lysates of cells containing the pLXIN vector, WT Nbs1 or the mutants revealed that the C-terminus of Nbs1 is required to immunoprecipitate E7 (Figure 2.3B). Importantly, we found that E7 was still able to co-immunoprecipitate with the Nbs1 mutant lacking the ATM binding domain, confirming our results with the ATM inhibitor (Figure 2.3B). The C terminus of Nbs1 contains an Mre11 binding domain (52, 53), in addition to an ATM binding site (38, 53, 61). To determine if the Mre11 binding domain of Nbs1 is required for immunoprecipitation of E7, we utilized a construct that is mutated in the Mre11 binding site. As shown in Figure 2.3C, mutation of the Mre11 binding site abrogated the ability of E7 to immunoprecipitate with Nbs1, suggesting that E7 interacts with Nbs1 through the Mre11 binding domain, though it is currently unclear if this occurs in a direct or indirect manner.

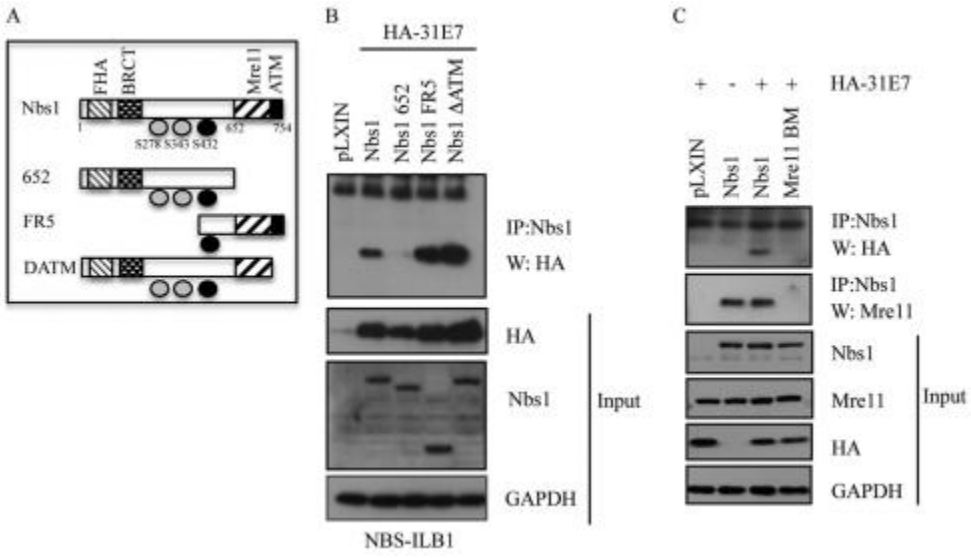

Figure 2.3. HPV 31 E7 interacts with Nbs1 through the Mre11 binding domain. (A) Schematic of Nbs1 constructs depicting relevant binding domains and phosphorylation sites. (B) NBS-ILB1 cells stably expressing pLXIN vector alone, or the indicted Nbs1 mutants were transiently transfected with HA-HPV31 E7. Immunoprecipitations were performed using an antibody to Nbs1, followed by Western blot analysis using an antibody to HA. Western blot analysis was performed on the indicated input lysates using antibodies to HA, Nbs1 and GADPH. (C) NBS-ILB1 cells stably expressing pLXIN vector alone, pLXIN-Nbs1, or a pLXIN-Nbs1 Mre11 binding mutant (Mre11 BM) were transiently transfected with HA-HPV31 E7. Immunoprecipitation of whole cell lysates was performed using an antibody to Nbs1 followed by Western blot analysis with an antibody to HA and Mre11. Western blot analysis was performed on the indicated input lysates using antibodies to HA, Nbs1, Mre11 and GADPH. IP=immunoprecipitation. W=immunoblotting. All results are representative of observations of three independent experiments.

To form the MRN complex, Nbs1 binds Mre11, which binds to Rad50 (40, 62). Since we were able to immunoprecipitate Nbs1 with E7, we next wanted to determine if E7 interacts with other components of the MRN complex. For this, we immunoprecipitated HA-tagged HPV31 E7 or TAP-tagged HPV16 E7 from lysates harvested from U20S cells, and performed Western blot analysis for Mre11 and Rad50. Interestingly, as shown in Figure 2.4A, we found that both HPV31 E7 and HPV16 E7 could immunoprecipitate with Rad50, but neither interacted with Mre11. However, we found that the presence of E7 did not disrupt formation of the MRN complex, as Nbs1 was still able to interact with Rad50 and Mre11 (Figure 2.4B). Rather, our results indicate that the presence of E7 increases the formation of the MRN complex, which may result from increased Mre11 expression. We also found that the formation of the MRN complex is not disrupted in HPV infected cells (Figure 2.8B) or cells stably expressing HPV31 E7 (data not shown).

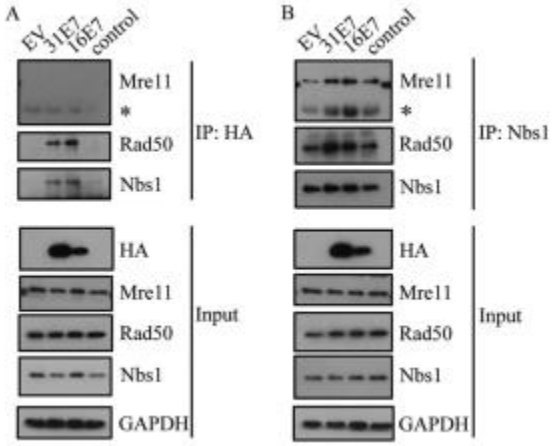

Figure 2.4. HPV E7 interacts with Nbs1 and Rad50, but not Mre11. (A) U20S cells were transiently transfected with vector alone (EV), HA-HPV31 E7, TAP-HPV16 E7, and immunoprecipitations of whole cell lysates were performed using an

HA antibody, followed by immunoblotting with Mre11, Rad50, and Nbs1 antibodies. (B) U20S cells were transiently transfected with EV alone, HA-HPV31 E7 or TAP-HPV16 E7. Immunoprecipitations were performed on lysates using an antibody to Nbs1, followed by Western blot analysis using antibodies to Mre11, Rad50, and Nbs1 antibodies. For (A) and (B), input lysates were analyzed by Western blot analysis using antibodies to HA, Mre11, Rad50 and Nbs1. GAPDH served as a loading control. IP=immunoprecipitation. * = antibody heavy chain. All results are representative of observations of three independent experiments.

Nbs1 is necessary for productive viral replication. Previously, we demonstrated that inhibition of ATM kinase activity has minimal effect on the ability of HPV to be maintained as an episome (12). However, in addition to being essential for ATM activation in response to DSBs, Nbs1 can also mediate ATR activation (63, 64), which could potentially be important for episomal maintenance. To examine the effect of Nbs1 depletion on episomal maintenance, we transduced HPV31 positive CIN612 9E cells with a scramble control shRNA or a previously validated Nbs1 shRNA (65), and generated stable cell lines. The cells were routinely passaged, and DNA and protein were harvested at every passage. Southern blot analysis was performed to examine the status of the episomal viral DNA. As shown in Figure 2.5 and Figure 2.12A, in two independently derived CIN612 9E lines, there was no significant difference in the ability of viral episomes to be maintained across passages in cells containing either the scramble control or the Nbs1 shRNA. Similar results were observed with four independent experiments (Figure 2.5B). These results mirror what we previously observed upon inhibition of ATM activity, and

indicate that Nbs1 is not necessary for episomal maintenance. In addition, we observed no effect of Nbs1 knockdown on Chk1 phosphorylation (data not shown).

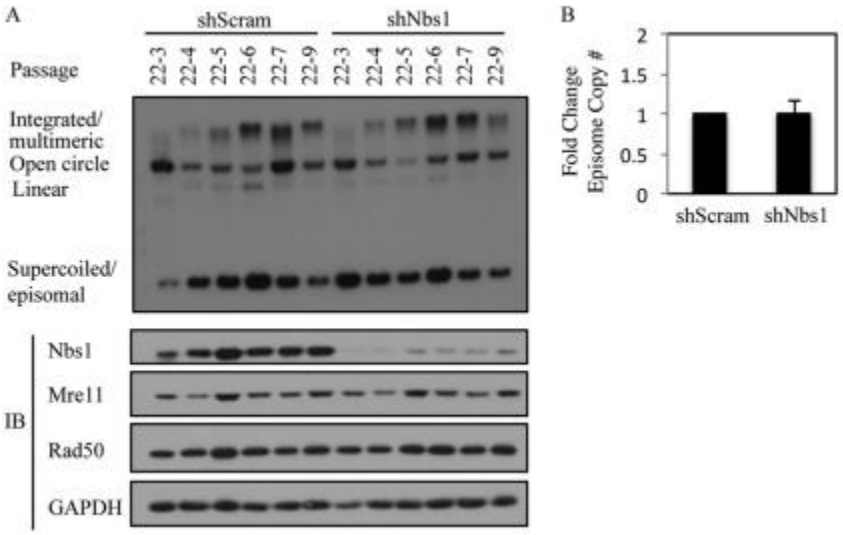

Figure 2.5. Nbs1 is not necessary for HPV31 genome maintenance. (A) DNA was isolated at the indicated passages from CIN612 9E cells stably maintaining a scramble shRNA (shScram) or Nbs1 shRNA (shNbs1) and analyzed by Southern blot analysis. Passage 22 (p22) represents the passage at which the CIN612 9E cells were transduced with the respective shRNAs. Each passage following transduction is represented by p22-#. Western blot analysis was performed on lysates harvested at each passage using an antibody to Nbs1 to demonstrate knockdown, as well as antibodies to Mre11 and Rad50. GAPDH served as a loading control. IB=immunoblotting. (B) Bar graph represents episome copy number quantified and averaged across the passages of Scramble shRNA (set at 1) containing cells compared to passages of Nbs1 shRNA containing cells. The data represent the average of four independent experiments. Densitometry was

performed using ImageJ software. The statistical analysis was assayed by 2-tailed t-test. Data=mean +/- standard error.

Since we previously found that ATM activity is necessary for productive viral replication, and given that Nbs1 is necessary for ATM activation in response to DSBs (32, 35, 36), we next wanted to determine the effect of Nbs1 depletion on productive replication. For this, CIN612 9E cells stably maintaining the scramble or Nbs1 shRNAs were induced to differentiate in high calcium medium, which activates the productive phase of the viral life cycle by 48 hours post-exposure (66). As shown in Figure 2.6A, Southern blot analysis for HPV DNA demonstrated that cells containing the Nbs1shRNA were greatly inhibited in their ability to undergo viral genome amplification compared to the scramble shRNA control, exhibiting a decrease in episomal copy number upon differentiation. This experiment was performed at least three times with three independently derived CIN612 9E lines with similar results. In addition, we found Nbs1 knockdown also decreased productive replication of human foreskin keratinocytes stably maintaining HPV31 genomes (HFK-31) after differentiation in methylcellulose (Figure 2.6C). Importantly, Nbs1 knockdown did not inhibit the ability of HPV positive cells to differentiate in high-calcium or methylcellulose as indicated by the expression of the differentiation-specific marker, involucrin (Figure 2.6B, D). In addition, as shown in Figure 2.7, Nbs1 knockdown had minimal effect on the level of cellular factors involved in replication and cell cycle regulation, including the S phase cyclins (cyclin A and cyclin E) and CDK (CKD2) (Figure 2.7A), as well as mitotic cyclin B, the M-phase

CDK (CDK1), and the CDC25C phosphatase (Figure 2.7B). In addition, both RPA and PCNA were maintained at similar levels in the Nbs1 shRNA cells compared to the scramble control (Figure 2.7C). These results suggest that the block in productive viral replication observed in response to Nbs1 knockdown was not due to alterations in cell cycle control or the lack of cellular factors directly required for viral replication. We also observed similar effects on productive viral replication following transient knockdown of Nbs1 for three days (data not shown). Overall, these results indicate that Nbs1 is specifically necessary for differentiation-dependent viral replication.

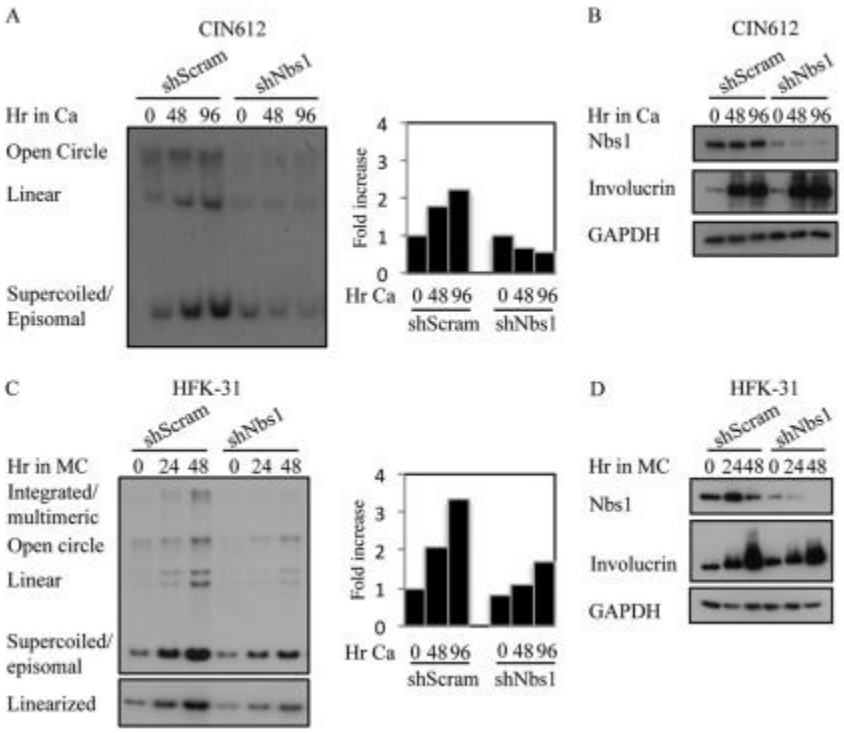

Figure 2.6. Nbs1 is necessary for productive viral replication. (A) DNA was harvested from CIN612 9E cells stably expressing a scramble shRNA or Nbs1 shRNA at T0 (undifferentiated) or after 48 and 96hr differentiation in high calcium

medium. Southern blot analysis was performed to analyze viral genome amplification. The bar graph represents quantification of the episome copy number present at each time point, relative to T0 shScramble, which was set to 1. Densitometry was performed using ImageJ. Ca=calcium. (B) Total protein was harvested from CIN612 9E shScramble and shNbs1 cells at T0 or after 48 and 96hr differentiation in high calcium. Western blot analysis was performed using antibodies to Nbs1, Involucrin, and GADPH as a loading control. (C) DNA was harvested from human foreskin keratinocytes stably maintaining HPV31 genomes (HFK-31) at T0 or after 24 and 48hr differentiation in methylcellulose (MC), and analyzed by Southern blot analysis for amplification of viral genomes. DNA samples were digested with BamHI (does not cut the viral genome, upper panel) or with HindIII to linearize viral genomes. The bar graph represents quantification of the episome copy number present at each time point, relative to T0 shScramble (set to 1). Densitometry was performed using ImageJ. (D) Western blot analysis was performed on lysates harvested from HFK-31 cells at T0 or after 24 and 48 hours differentiation in methylcellulose using antibodies to Nbs1 and Involucrin. GAPDH was used as a loading control. All results are representative of observations of four or more independent experiments.

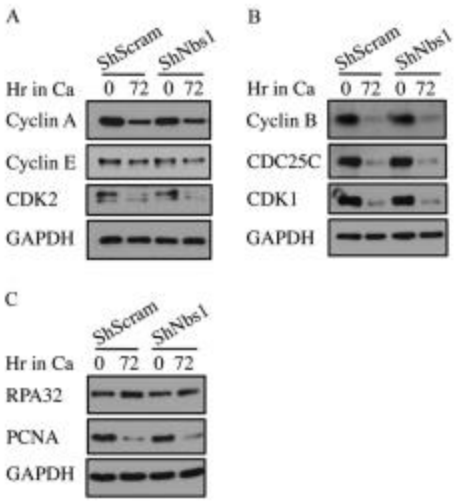

Figure 2.7. Levels of cell cycle or replication proteins are not affected by Nbs1 knockdown. Western blot analysis was performed on lysates harvested from CIN612 9E cells stably maintaining the scramble (shScram) or Nbs1 shRNAs at T0, or after 72 hours of differentiation in high calcium medium using antibodies to (A) Cyclin A, Cyclin E, and CDK2; (B) Cyclin B, CDC25C, and CDK1; and (C) RPA, as well as PCNA. GAPDH served as a loading control. All results are representative observations of at least three independent experiments. Ca=calcium.

The MRN complex is disrupted upon Nbs1 knockdown. As Nbs1 functions in a complex with Mre11 and Rad50, we next wanted to determine if Nbs1 depletion affected the levels of Mre11 and Rad50. As shown in Figures 5A and 8A, Nbs1 knockdown had little effect on the levels of Mre11 or Rad50, as measured by Western blot analysis, consistent with previous studies (58). Although Rad50 levels appear to decrease after 72 hours in high calcium (Figure 2.8A), this result was not consistently observed. Since Nbs1 contains a nuclear localization signal and is

necessary for the localization of Mre11 and Rad50 to the nucleus (52, 67), we next wanted to determine if Nbs1 depletion affected Mre11 and Rad50 localization. As shown in Figure 2.8B, CIN612 9E cells exhibited increased levels of Mre11, Rad50 and Nbs1 in the nucleus compared to uninfected HFKs, indicating increased MRN complex formation, which is consistent with our immunoprecipitation results (Figure 2.4B). However, upon depletion of Nbs1, we observed a dramatic decrease in levels of Mre11 and Rad50 in the nucleus of CIN612 9E cells and a marked re-localization to the cytoplasm (Figure 2.8C), indicating disruption of the MRN complex, as previously shown in NBS fibroblasts (40). These results raise the possibility that formation of the MRN complex, rather than Nbs1 alone, is crucial to productive viral replication. To test this, we examined the effect of MIRIN, an inhibitor of Mre11 nuclease activity, on productive replication of CIN612 9E cells. As shown in Figure 2.8D, inhibition of Mre11 by MIRIN resulted in a decreased ability of viral episomes to amplify upon differentiation, similarly to Nbs1 depletion. Similar results were observed in three independent experiments. Overall, these results indicate that the MRN complex is required for productive viral replication.

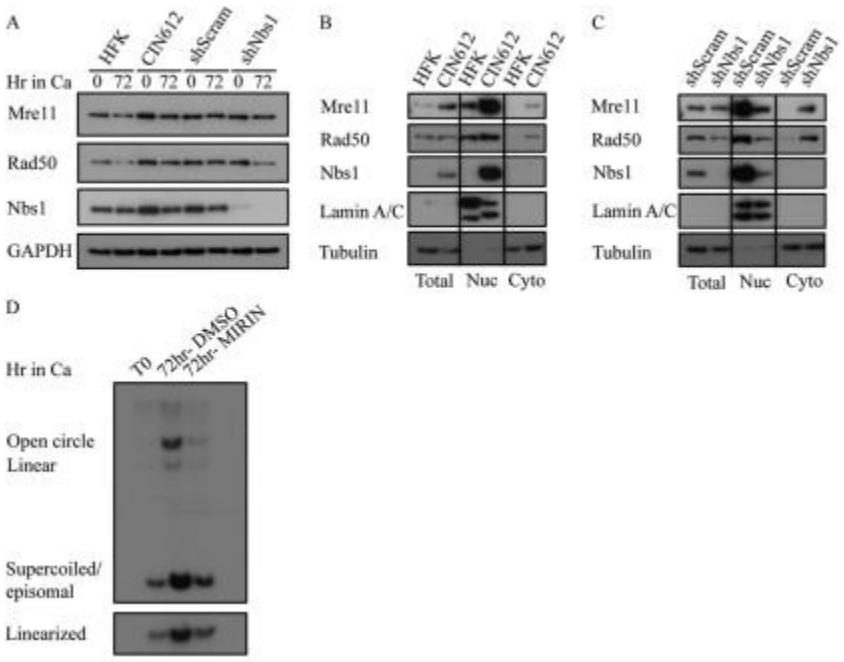

Figure 2.8. Nbs1 knockdown disrupts MRN complex formation. (A) Whole cell lysates were harvested from HFK, CIN612, and CIN612 9E cells stably expressing shScramble or shNbs1 at T0 and after 72hr differentiation in high calcium medium (Ca). Immunoblotting was performed using Mre11, Rad50 and Nbs1 antibodies. GAPDH was used as a loading control. (B) Total, nuclear (nuc), and cytoplasmic (cyto) lysates were harvested from HFK and CIN612 cells. Immunoblotting was performed using Mre11, Rad50 and Nbs1 antibodies. (C) Total, nuclear (nuc), and cytoplasmic (cyto) lysates were harvested from stable CIN612 9E shScramble and CIN612 9E shNbs1 cells. Immunoblotting was performed using Mre11, Rad50 and Nbs1 antibodies. For (B) and (C) Lamin A/C and tubulin were used to confirm nuclear and cytoplasmic fractionation, respectively. (D) DNA was harvested from CIN612 9E cells at T0 and after 72hr differentiation in high calcium medium with DMSO as a vehicle control or 50uM of the Mre11 inhibitor MIRIN. Southern blot

analysis was performed to analyze viral genome amplification of DNA digested with BamHI (non-viral genome cutter, upper panel) or HindIII (cuts viral genome once, lower panel). All results are representative of observations of two or more independent experiments.

Nbs1 knockdown affects the localization of Mre11, Rad50 and the HR factor Rad51 to HPV DNA foci. Since we previously observed that MRN components co-localize with HPV DNA foci(18), we next wanted to determine if Nbs1 knockdown affected the localization of Mre11 and Rad50 to sites of HPV DNA replication. We performed Fluorescence in Situ Hybridization (FISH) for HPV DNA coupled with immunofluorescence for Mre11 and Rad50, as described previously (3, 18). As shown in Figure 2.9, in undifferentiated CIN612 9E cells containing the scramble shRNA, we found Mre11 and Rad50 co-localized with viral genomes in ~44% $\pm$ 5.6 and 41% $\pm$ 0.78 of cells containing HPV DNA foci, respectively (Figure 2.9A-D). Upon differentiation in high calcium, this number significantly increased, with Mre11 and Rad50 co-localizing with viral genomes in 90 $\pm$ 5.3% and 86 $\pm$ 0.98% of cells positive for HPV DNA foci, respectively. Consistent with viral genome amplification, the size of the Mre11/Rad50/HPV DNA foci increased upon differentiation. In contrast, Nbs1 knockdown resulted in a significant decrease in the localization of Mre11 and Rad50 to HPV DNA foci in undifferentiated cells, with Mre11 and Rad50 localized to viral genomes in 9% $\pm$ 1.3 and 8% $\pm$ 4.3 of HPV DNA foci positive cells, respectively (Figure 2.9A-D). Although an increase in Mre11 and Rad50 localization to viral genomes was observed upon differentiation, this was

significantly less compared to the scramble control cells (Mre11 20% ± 17, Rad50 14% ± 5.5). In addition, consistent with a block in viral genome amplification, the size of the HPV DNA/Mre11/Rad50 foci did not increase upon differentiation. These results suggest that Nbs1 may contribute to productive replication through the localization of MRN components to viral genomes.

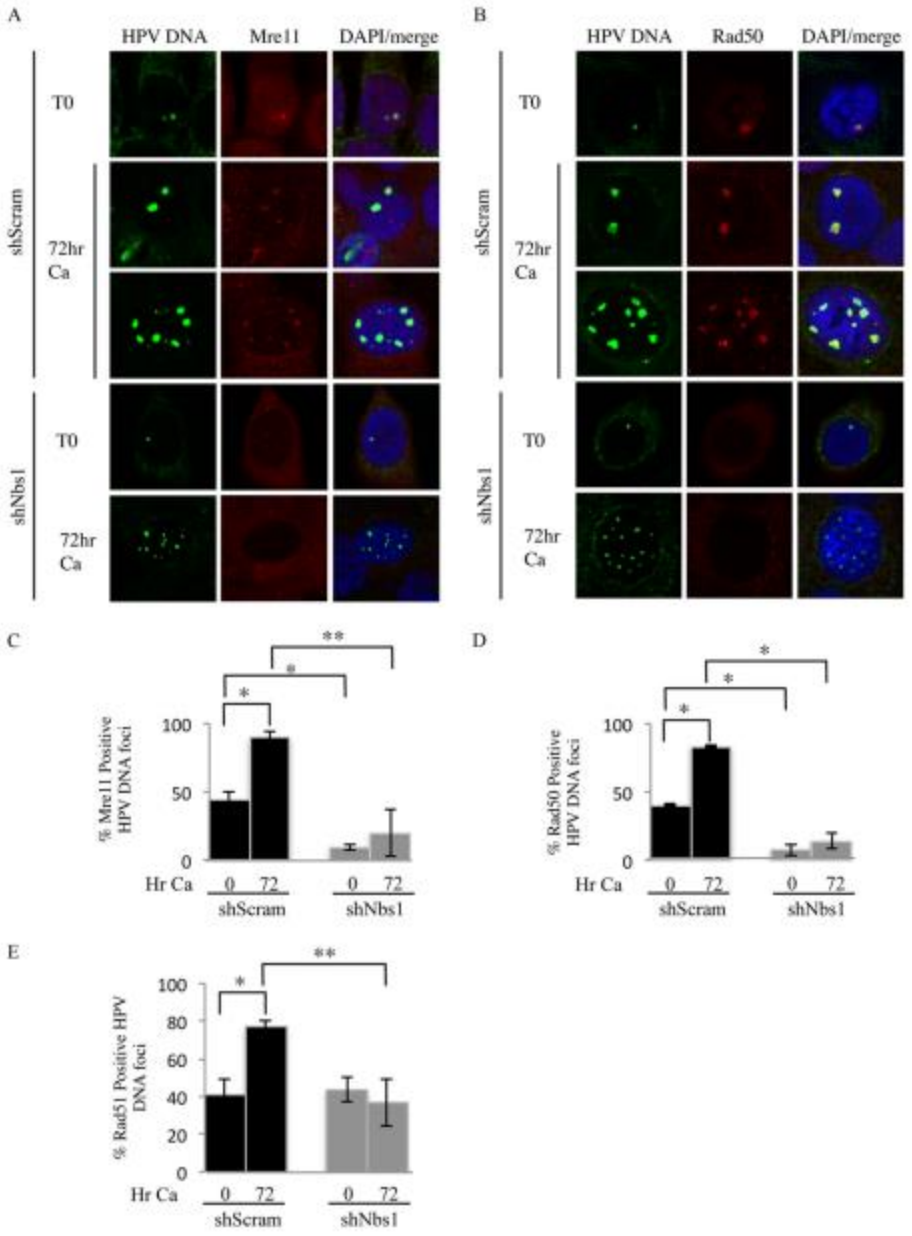

Figure 2.9. Nbs1 knockdown results in decreased localization of Mre11, Rad50 and Rad51 to viral genomes. Immunofluorescence (IF) for (A) Mre11 (red) and (B) Rad50 (red), followed by fluorescence in site hybridization (FISH) for HPV DNA (green) was performed on CIN612 9E cells stably maintaining the scramble or Nbs1 shRNAs at T0 (undifferentiated), as well as after 72 hours differentiation in high calcium medium. Cellular DNA was counterstained with DAPI. (C, D) Bar graphs represents quantification of percent of HPV DNA foci positive cells that were also positive for (C) Mre11 and (D) Rad50. (E) Quantification of percent of cells positive for HPV DNA foci by FISH that were also positive for Rad51 by IF in CIN612 9E-shScram and –shNbs1 cells at T0, as well as after 72 hours of differentiation in high calcium. The data represent the average of at least three independent experiments. The statistical analysis was assayed by 2-tailed t-test. Data=mean +/- standard error. ** = p-value less than 0.05. * = p-value less than 0.01. Ca=calcium.

Since the MRN complex is important in facilitating ATM-mediated HR repair (22, 39), we examined the effect of Nbs1 knockdown on the localization of the principal HR factor Rad51, a recombinase we previously showed localized to HPV replication foci (18). Interestingly, in undifferentiated cells, we found no difference in Rad51 localization to HPV DNA foci between the Scramble control (~41% $\pm$ 8.4) and Nbs1 knockdown (~44% $\pm$ 6.25) (Figure 2.9E). Upon differentiation, however, while Rad51 localization to HPV DNA foci significantly increased in the Scramble control cells (~77% $\pm$ 3.4), no increase was observed in the Nbs1 knockdown cells, with Rad51 localizing to ~37% $\pm$ 12.8 of cells containing HPV DNA foci. Overall, these

results indicate that Nbs1 is required for the localization of Mre11 and Rad50 to viral genomes, and suggest that productively replicating genomes require Rad51 activity, which may rely on an intact MRN complex.

Nbs1 knockdown moderately affects phosphorylation of ATM and Chk2 in HPV positive cells. Nbs1, as part of the MRN complex, is an essential part of the DDR, acting upstream to activate ATM, as well as downstream in the intra-S phase checkpoint, and in the repair of DSBs through HR (13). To determine if Nbs1 contributes to productive viral replication through facilitating ATM activation, we examined the effect of Nbs1 depletion on ATM and Chk2 phosphorylation in CIN612 9E cells. We first examined the effect of stable Nbs1 knockdown on ATM pSer1981 (pATM) and Chk2 pThr68 (pChk2) in undifferentiated cells over several passages. As shown in Figure 2.10, pATM and pChk2 were both decreased to a certain extent in response to Nbs1 depletion. While the decrease in pATM relative to total ATM ranged from 1.3-2-fold over the indicated passages, the decrease in Chk2 phosphorylation relative to Chk2 was more dramatic, ranging from a 1.2 to 8.3-fold difference in this particular line (Figure 2.10). We previously showed that Chk2 activation is dependent on ATM activity in HPV positive cells (12), indicating that a small decrease in ATM phosphorylation is sufficient to induce significant changes in downstream targets. These results suggest that Nbs1 may affect viral genome amplification through its affects on ATM and Chk2, both of which are necessary for productive replication.

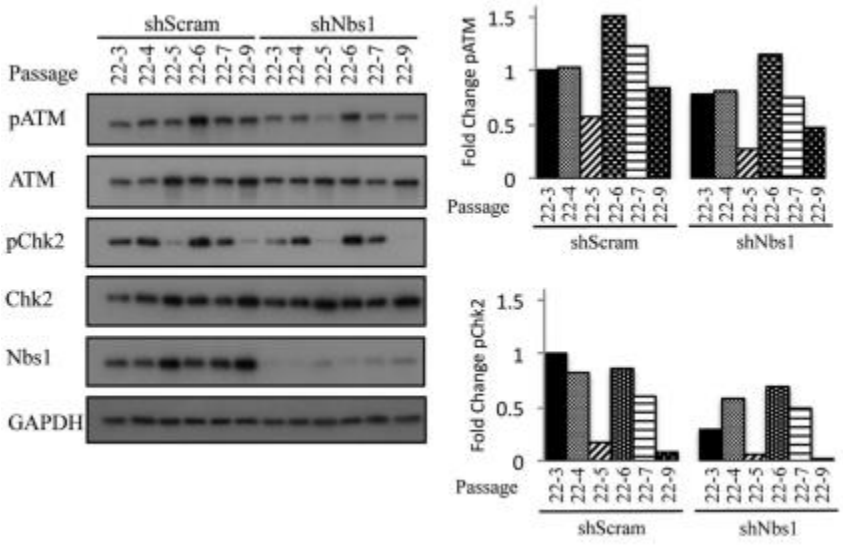

Figure 2.10. Phosphorylated ATM and Chk2 levels are decreased variably in response to Nbs1 knockdown. Whole cell lysates were harvested from stable CIN612 9E shScramble and shNbs1 cells at the indicated passages. Passage 22 (p22) represents the passage at which the CIN612 9E cells were transduced with the respective shRNAs. Each passage following transduction is represented by p22-#. Lysates were immunoblotted with antibodies to phosphorylated ATM (Ser1981) (pATM) and Chk2 (Thr68) (pChk2), as well as total ATM, Chk2, and Nbs1. GAPDH served as a loading control. Protein levels were quantified using ImageJ, with phosphorylated protein levels first normalized to total levels and then GAPDH. Bar graphs represent the fold changes compared to the first passage of the shScramble cells for this representative experiment, which is set at 1. Results shown are representative of observations of three independent experiments.

We next determined if Nbs1 knockdown results in a further decrease in pATM and pChk2 upon differentiation. As shown in Figure 2.11A and B, in this particular passage (p22-6) at time zero (undifferentiated), the levels of pATM in CIN612 9E-

shNbs1 cells were decreased only 1.3-fold compared to scramble control, while pChk2 remained essentially unchanged. Upon differentiation, although Nbs1 levels decreased in CIN612-shNbs1 cells, there was no further decrease in pATM levels compared to total, and these cells exhibited only a moderate 1.6-fold decrease in pATM upon differentiation compared to scramble control. Despite the relatively high level of pATM, however, viral genome amplification was still diminished in shNbs1 cells (Figure 2.11C). Interestingly, the decrease in pATM levels in shNbs1 cells upon differentiation did not correlate with decreased pChk2 levels. Rather, pChk2 levels increased relative to total Chk2 in CIN612-shNbs1 cells, as well as in CIN612-shScramble and CIN612 control cells (Figure 2.11B). An increase in pChk2 was observed upon differentiation across multiple passages (Figure 2.12D), as well as with HFK-31-shScramble and –shNbs1 cells suspended in methylcellulose (data not shown). While the decrease in pATM and pChk2 observed in undifferentiated cells suggests that Nbs1 contributes to their activation in HPV positive cells, the relatively high levels of pATM and pChk2 observed upon differentiation in shNbs1 cells potentially indicates an alternative mechanism for ATM activation, independent of the MRN complex. Overall, these data offer support that Nbs1 contributes to productive replication at least in part through a mechanism that is independent of promoting ATM activation. Nbs1 may serve as a downstream effector of ATM activity through phosphorylation, and/or ensure the localization of repair factors to viral genomes that are required for efficient DNA synthesis, which could potentially occur in a recombination-dependent manner.

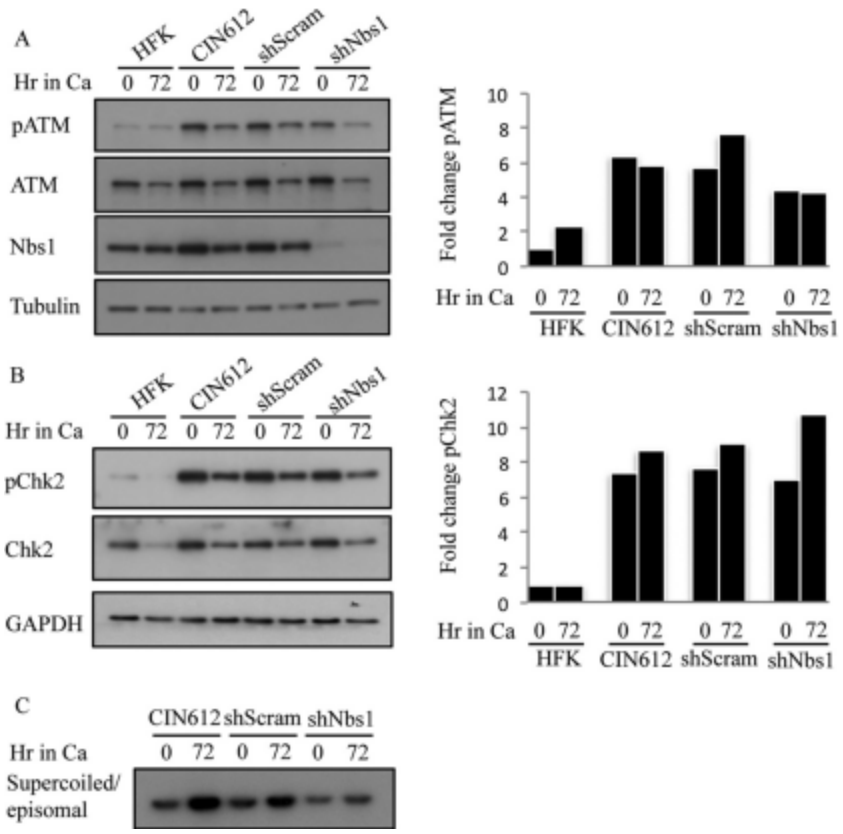

Figure 2.11. Phosphorylation of ATM and Chk2 is maintained with Nbs1 knockdown upon differentiation. (A) Whole cell lysates were harvested from HFK, CIN612 9E, as well as CIN612 9E cells stably expressing shScramble or shNbs1 cells at T0 and 72hr after differentiation in high calcium medium. Immunoblotting was performed using antibodies to phosphorylated ATM (Ser1981) (pATM), total ATM and Nbs1. Tubulin was used as a loading control. Protein levels were quantified using ImageJ, with phosphorylated protein levels first normalized to total levels and then to tubulin. Levels for this representative experiment are graphed as fold change compared to the T0 HFK sample, which is set to 1. (B) Whole cell lysates were harvested from HFK, CIN612 9E, CIN612 9E shScramble, and CIN612 9E shNbs1 cells at T0 and 72hr after differentiation in high calcium. Immunoblotting

was performed using antibodies to phosphorylated Chk2 (Thr68) (pChk2), total Chk2 and Nbs1. GAPDH was used as a loading control. Protein levels were quantified using ImageJ as indicated above. Shown in a representative experiment where levels are graphed as fold change compared to the T0 HFK sample, which is set at 1. (C) DNA was harvested from CIN612 9E, CIN612 9E shScramble, and CIN612 9E shNbs1 cells at T0 and after 72hr differentiation in high calcium, and linearized by digestion with HindIII. HPV episomes were visualized via Southern blot analysis. Results shown are representative observations of four or more independent experiments.

Levels of pATM and pChk2 do not influence productive viral replication. While we previously found that ATM and Chk2 activity is necessary for productive replication (12), it is unclear if higher levels of pATM and/or pChk2 could influence the ability of HPV to productively replicate. As shown in Figures 10 and 12B, in two independently derived CIN612-shScramble and shNbs1 lines, we have found that the levels of pATM and pChk2 fluctuate over time, with or without depletion of Nbs1. Similar results were observed for phosphorylated SMC1 S966 (pSMC1), a target of ATM that requires Nbs1 for phosphorylation and is involved in the intra-S phase checkpoint (Figure 2.12B) (68-70). To determine if the level of pATM and pChk2 in undifferentiated cells affects productive replication, and to further examine the effect of Nbs1 depletion on pATM and pChk2 levels upon differentiation, we examined viral genome amplification in three different passages of CIN612 9E cells stably expressing the scramble or Nbs1 shRNA (Figure 2.12C). We compared passages

19-2, 19-3 and 19-6, which in undifferentiated cells, Nbs1 knockdown resulted in a 2.2-, 1.75- and 1.53- fold decrease in pATM, respectively, and a 1.4-, 2.2-, and 1.1-fold decrease in pChk2, respectively (Figure 2.12B). We found that even though the p19-6 Scramble control cells (undifferentiated) exhibited higher levels of pATM compared to p19-2 and p19-3 (Figure 2.12B), productive viral replication was not enhanced (Figure 2.12C). Likewise, in the Nbs1shRNA cells, the higher level of pATM and pChk2 present in the p19-6 undifferentiated cells was not sufficient to allow for productive viral replication. In addition, as shown in Figure 2.12D, Nbs1 knockdown had no significant affect on pATM and pChk2 levels upon differentiation when averaged across these three passages, and we observed similar results for pSMC1 (data not shown). The lack of productive replication in Nbs1 knockdown cells, despite maintenance of pATM and pChk2, furthers suggests that Nbs1 has a multifaceted role in promoting productive replication, one that is more complex than functioning solely to activate ATM.

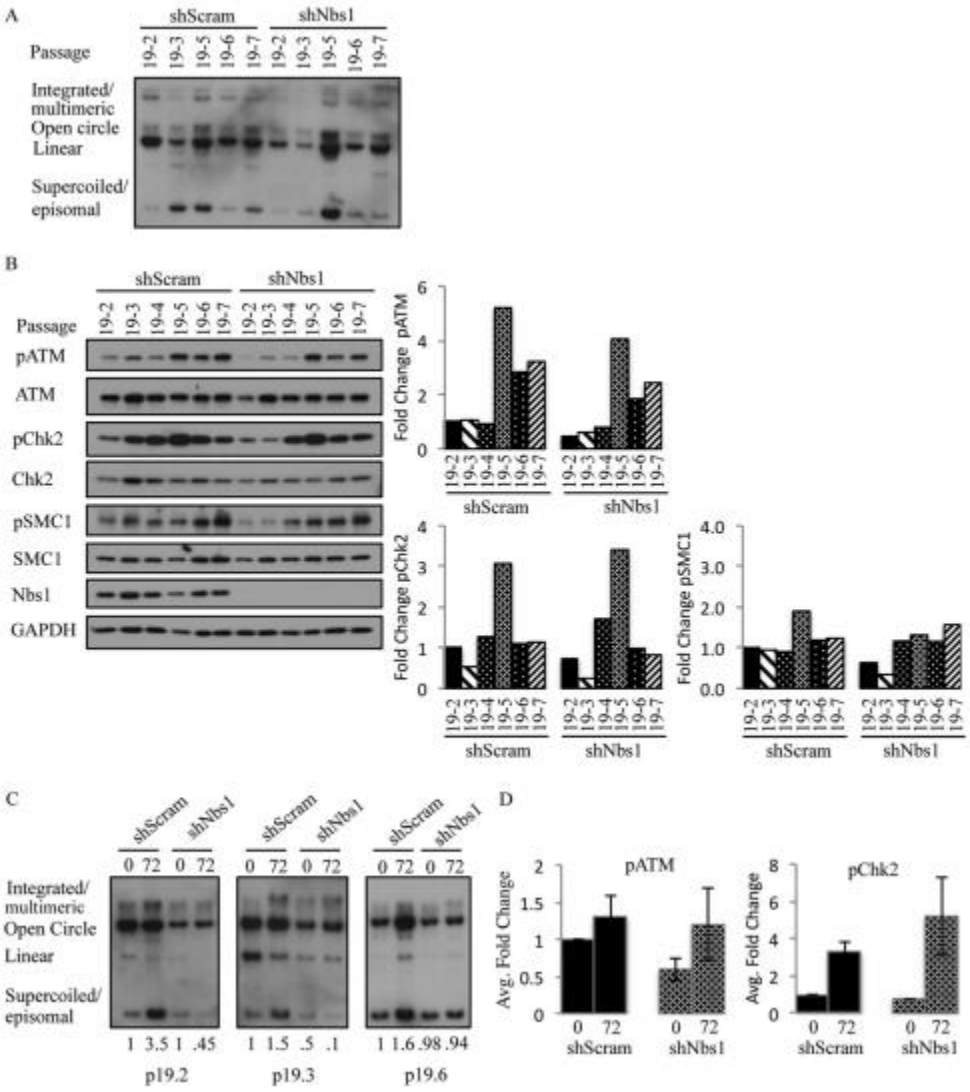

Figure 2.12. Levels of phosphorylated ATM and Chk2 do not influence viral genome amplification. (A) DNA was harvested from CIN612 9E cells stably expressing shScramble and shNbs1 at the indicated passages, and digested with BamHI (non-viral genome cutter). HPV genomes were visualized via Southern blot analysis. Passage 19 (p19) represents the passage at which the CIN612 9E cells were transduced with the indicated shRNAs. P19-# indicates that passage at which DNA and protein were harvested following transduction. (B) Whole cell lysates were harvested from CIN612 9E shScramble and shNbs1 cells at the indicated passages,

and immunoblotting was performed with antibodies to phosphorylated ATM (Ser1981) (pATM), phosphorylated Chk2 (Thr68) (pChk2), phosphorylated SMC1 (Ser966) (pSMC1) and total ATM, Chk2, SMC1 and Nbs1. GAPDH was used as a loading control. Protein levels were quantified using ImageJ, with phosphorylated protein levels first normalized to total levels and then GAPDH. Levels are graphed as fold change compared to the first passage (p19-2) of the shScramble cells, which is set to 1. (C) DNA was harvested from p19-2, p19-3 and p19-6 CIN612 9E shScramble and shNbs1 cells at T0 and after differentiation for 72hr in high calcium medium. Southern blot analysis was performed to analyze viral genome amplification. Levels of HPV episomes were quantified using Image J; episome amounts are shown relative to the T0 shScramble, which is set to 1. (D) Protein was harvested from p19-2, p19-3 and p19-6 CIN612 9E shScramble and shNbs1 cells at T0 as well as after 72hr differentiation in high calcium medium. Western blot analysis was performed using antibodies to phosphorylated ATM (S1981) (pATM), and phosphorylated Chk2 (Thr68) (pChk2). GAPDH served as a loading control. Protein levels were quantified using ImageJ, as described above. Graphed is the average fold change in pATM and pChk2 levels across the three passages in undifferentiated shScram and shNbs1 cells compared to cells differentiated in high calcium for 72hr. Fold change is shown relative to shScram T0, which is set to 1. Results shown are representative of observations of three or more independent experiments.

DISCUSSION

Previously, we demonstrated that MRN components localize to sites of HPV DNA synthesis (18). We have now found that Nbs1 is necessary for productive replication, though exactly how Nbs1 contributes to efficient viral DNA synthesis is unclear. Nbs1, as part of the MRN complex, acts as a sensor of DNA breaks and also regulates activation of ATM (32, 39). We have found that depletion of Nbs1 disrupts formation of the MRN complex, resulting in re-localization of Mre11 and Rad50 to the cytoplasm. This, coupled with the finding that the nuclease activity of Mre11 is also required for productive replication, suggests a key role for the MRN complex during the productive phase of the viral life cycle.

One of the simplest explanations for how Nbs1 contributes to productive viral replication is through facilitating activation of ATM. However, while Nbs1 knockdown did affect the levels of pATM and pChk2 to some extent, pATM and pChk2 remained quite high for most passages, especially upon differentiation. Despite the presence of pATM and pChk2, however, a defect in HPV genome amplification was consistently observed. These results suggest that Nbs1, while certainly influencing activation of ATM in HPV-infected cells, is likely playing a role outside of its function as an upstream regulator of ATM activation to drive productive viral replication.

There are several possible ways by which Nbs1 could contribute to viral replication independently of activating ATM. Nbs1 is a target for phosphorylation by ATM on Ser343, and this phosphorylation is necessary for activation of the intra-S phase checkpoint (71), which serves to slow down cellular DNA replication in response to DNA damage (70). However, while phosphorylation of SMC1, a key

regulator in this checkpoint (68, 69), was decreased upon Nbs1 knockdown, pSMC1 levels exhibited little change upon differentiation, suggesting that a block in the intra-S phase checkpoint was not responsible for the abrogation of productive replication. Nbs1 has also been implicated in DNA damage-induced apoptosis (72). While we have shown that caspase activation is necessary for productive viral replication (66), Nbs1 knockdown had no effect on caspase activation in differentiating cells (data not shown). This is not surprising, as pChk2 levels were not affected upon differentiation by Nbs1 depletion, and we previously found Chk2 activation to be necessary for caspase activation (66).

As mentioned, downstream of ATM activation, Nbs1 and the MRN complex also assist in the repair of DNA breaks, primarily through homologous recombination (HR) when cells are in S or G2 phases of the cell cycle (32). Nbs1, as part of this complex, has specific functions that contribute to efficient HR repair. Nbs1 contains protein-protein interaction motifs important for the localization of MRN to nuclear foci (52, 73, 74), as well as the recruitment of CtIP (CtBP-interacting protein) to DNA DSB ends for end processing and HR (22, 75-77). In addition to two ATM phosphorylation sites (S278, S343) (71, 78, 79), Nbs1 is also phosphorylated by CDK2 on Ser432, which stimulates MRN-dependent conversion of DSBs into structures recognized by HR for repair (80, 81). Furthermore, recent studies indicate that Nbs1 may be required for Rad51 localization to RPA-coated DNA, and the restart of stalled replication forks (81, 82).

Our finding that productive viral replication requires ATM activity and Nbs1, coupled with the localization of the MRN complex and the HR factors Rad51 and

Brca1 to sites of viral genome synthesis (18, 83), suggests that recombination-dependent repair may play a role in productive viral replication. We have found that Mre11, Rad50 and Rad51 increase in localization to viral genomes upon differentiation, and that this is abrogated in the absence of Nbs1. These results suggest that these repair and recombination factors are required at viral DNA to ensure efficient productive replication, possibly through facilitating HR. In support of this, preliminary studies from our lab indicate that Rad51 is necessary for productive viral replication (W. Chappell and C. Moody, unpublished). Whether Nbs1 recruits Rad51 to viral DNA, or if productive replication results in HR structures that are processed by MRN then bound by Rad51, is currently unclear. The massive amplification of HPV genomes upon differentiation could result in DSBs or stalled replication forks that require ATM-mediated HR to re-start (84), and it will be important to examine the effect of ATM inhibition, as well as depletion of Nbs1 on the formation of replication intermediates to assess this possibility, as has been done recently for SV40 (85).

Curiously, we found that Mre11 and Rad50 localize to viral genomes in ~40% of cells containing HPV DNA foci in undifferentiated Scramble control cells, despite Nbs1 not being required for episomal maintenance. The possibility exists that the MRN complex contributes to viral replication in undifferentiated cells, but in its absence, replication proceeds efficiently enough such that episomal copy number and maintenance are not affected at a discernable level. This may also apply to Rad51, which we also found localized to viral genomes in a similar percentage of undifferentiated HPV DNA foci positive cells. In contrast to Mre11 and Rad50

though, Nbs1 depletion did not affect Rad51 localization to viral genomes in undifferentiated cells. However, recent studies have shown that in the absence of Nbs1, Rad51 foci can serve as markers of HR substrates, such as replication intermediates, rather than serve as an indicator of HR activity (86). Understanding the contribution of Rad51 and recombination to maintenance replication, in addition to productive replication, will be important areas of future research.

The finding that ATM and Chk2 are phosphorylated at high levels in differentiating cells upon Nbs1 knockdown suggests that either ATM activation occurs in an MRN-independent manner, or that an alternative mode of ATM activation is stimulated in the presence of decreased Nbs1. The ATM interactor (ATMIN) protein is required for ATM signaling in an Nbs1-independent manner in response to hypotonic stress, as well as inhibitors of replication such as hydroxyurea (87). Nbs1 knockdown could potentially increase the flux through the ATMIN-dependent arm of the ATM signaling pathway (87, 88), maintaining phosphorylation of ATM targets. The DNA repair protein 53BP1, which is expressed at high levels in HPV positive cells (18), has also been shown to be a mediator of ATM function (89, 90), with its effects on ATM activity enhanced in situations in which the MRN complex is present at low levels (90). Activated STAT5 has been shown to be necessary for ATM activation in HPV-positive cells (16), though whether this occurs in an MRN-dependent manner is unclear. Regardless, the maintenance of ATM signaling upon differentiation is not sufficient to drive productive viral replication, as the decreased levels of Nbs1 still act as a barrier to viral replication. Understanding

the role of the MRN complex in the regulation of ATM activity in HPV-infected cells will be the focus of future investigations.

Our studies indicate that E7 expression is sufficient to increase protein levels of MRN components, as well as that of other repair factors, such as Chk2. Interestingly, E7 significantly increased only the transcript levels of Mre11, which may in turn serve to stabilize protein levels of Nbs1 and Rad50 in infected cells. We previously showed that HPV positive cells exhibit increased levels of key proteins involved in HR repair (18), including Rad51 and Brca1, and preliminary studies indicate this occurs in an E7- and E2F-dependent manner (W. Chappell, B. Johnson, and C. Moody, unpublished). Taken together, these results suggest that E7 may increase levels of proteins involved in the DNA damage response to support viral replication. Recent studies by Hong and Laimins demonstrated the protein levels of ATM, Chk2, Brca1 and Rad51 decreased upon STAT5 depletion (16). Deregulation of E2F and STAT5 activity may be necessary to ensure sufficient levels of DNA repair genes that drive viral replication in response to E7-, or potentially E6- and E1-induced DNA damage (8, 15, 17, 83).

We have found that E7 immunoprecipitates with Nbs1, but in a manner independent of ATM. Interestingly, we found that E7 also immunoprecipitates with Rad50, but not Mre11. Nbs1 has been shown to be capable of forming a complex with Rad50 (91), and it will be important to determine if E7 binds directly with either of these MRN components, as has been shown for HSV UL12 (44). We have found that immunoprecipitation of Mre11 does not pull down E7 (data not shown), suggesting that at least two, possibly three, complexes exist in HPV-infected cells:

Mre11/Rad50/Nbs1 and E7/Nbs1/+/-Rad50. E7 may form a complex with Nbs1 in the nucleus, regulating its stability when not bound to Mre11. Several studies indicate the activity of Nbs1 is regulated by post-translational modifications, including acetylation in addition to phosphorylation, and E7 may contribute to this regulation through multiple mechanisms. E7 increases levels of SIRT1 (93), which deacetylates Nbs1 to allow its phosphorylation by ATM on S343 (92). E7 decreases the acetyltransferase activity of PCAF (94), which has been shown to acetylate Nbs1 and block phosphorylation (92). In addition, E7 maintains CDK2 activity (5, 95, 96), and may target Nbs1 for phosphorylation through binding, potentially regulating HR repair in HPV infected cells.

In summary, our studies demonstrate that HPV utilizes Nbs1 and the MRN complex for efficient productive replication. Nbs1 knockdown does not significantly affect the phosphorylation of ATM and Chk2 upon differentiation, suggesting that Nbs1 may contribute to efficient productive replication outside of its ability to facilitate ATM activation, potentially through the localization of Mre11, Rad50 and Rad51 to viral genomes. In addition, we have shown that E7 affects expression of DNA repair factors that are required for productive replication. E7 may further modulate the DNA damage response and drive viral replication through interactions with components of the MRN complex.

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CHAPTER 3: HPV31 UTILIZES THE ATR-CHK1 PATHWAY TO MAINTAIN ELEVATED RRM2 LEVELS AND A REPLICATION-COMPETENT ENVIRONMENT IN DIFFERENTIATING KERATINOCYTES

OVERVIEW

The life cycle of human papillomaviruses (HPV) is intimately linked to the differentiation status of the host cell, with productive viral replication and virion formation restricted to the uppermost layers of the stratified epithelium. While cell cycle re-entry upon differentiation provides an environment conducive to rapid amplification of viral genomes, the mechanisms by which HPV ensures an adequate supply of cellular substrates for viral DNA synthesis are unclear. RRM2 is a key component of the ribonucleotide reductase (RNR) complex, which is required for *de novo* synthesis of dNTPs, the building blocks of nucleic acid. In this study, we have found that HPV31 positive cells exhibit increased RRM2 levels accompanied by elevated dNTP pools. RRM2 expression is regulated in an E2F1-dependent manner, and expression of E7 is sufficient to increase RRM2 at the transcript and protein level. Loss of RRM2 expression blocks productive replication, suggesting that HPV requires increased RRM2 to provide dNTPs for accelerated viral DNA synthesis in differentiating cells. Interestingly, we have found that RRM2 levels are regulated in HPV31 positive cells by activation of the ATR-Chk1-E2F1 DNA damage response pathway, which is essential to combat replication stress upon entry into S-phase.

Importantly, ATR and Chk1 have recently been shown to be required for productive HPV31 replication. Overall, our studies suggest that E7-induced cycle re-entry upon differentiation triggers replication stress that activates ATR and Chk1. In turn, increased levels of E2F1 drive RRM2 expression to facilitate productive viral replication.

INTRODUCTION

Human papillomaviruses (HPV) are small, double stranded DNA viruses that exhibit a strict tropism for epithelial cells. A subset of approximately 15 HPVs, termed high-risk (HR) (e.g. HPV16, 18, 31, 45), is the causative agent of cervical cancer, and is also associated with other genital malignancies, as well as an increasing number of head and neck cancers (1). While the three licensed HPV vaccines show great efficacy and offer great promise in reducing the number of deaths due to cervical cancer, these vaccines are not therapeutic. Identifying cellular pathways HPV commandeers to promote replication may identify potential therapeutic targets to limit viral replication and block disease progression.

The limited coding capacity of the HPV genome renders the virus reliant on cellular factors for viral replication. The viral life cycle is intimately linked to the differentiation status of the stratified epithelium and is characterized by three distinct phases of replication (2). HPV infects the actively dividing basal cells of the stratified epithelium, where viral episomes undergo limited amplification to 50-100 copies per cell. Viral genomes are then maintained in these undifferentiated cells by replicating once per cell cycle along with cellular DNA. Upon epithelial differentiation, the productive phase of the viral life cycle is triggered, resulting in amplification of viral

genomes to 1000s of copies per cell, late gene expression and virion production. Paradoxically, these late viral events occur in cells that normally would have exited the cell cycle. HPV circumvents this problem largely through E7's ability to target the tumor suppressor Rb for degradation, resulting in the release of active E2F transcription factors that drive expression of genes required for entry into the cell cycle (3). Traditionally, cell cycle re-entry has been thought to provide an S phase environment conducive to rapid amplification of viral genomes. However, more recent studies indicate that productive replication occurs post-cellular DNA synthesis, in a prolonged G2-like phase (4). Therefore, HPV must have evolved means to provide cellular factors required for productive replication outside of S phase.

One of the most important resources HPV needs to acquire for productive replication is a sufficient supply of deoxyribonucleotide triphosphates (dNTPs), the building blocks for DNA synthesis. However, it is unclear how dNTP pools are maintained in a differentiating environment where infected cells are no longer dividing. The ribonucleotide reductase (RNR) enzyme complex is required for *de novo* synthesis of dNTPs (5). RNR is the rate-limiting enzyme for dNTP synthesis, catalyzing the conversion of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates (dNDP). Subsequent phosphorylation of dNDPs by the nucleoside diphosphate kinase provides balanced pools of dNTPs utilized for DNA replication, as well as repair. The active form of RNR is a tetramer composed of two identical large subunits of RMM1, and either two identical small subunits of RRM2, or an alternative subunit p53R2, which is p53-responsive (5, 6). In

proliferating cells, the RRM1-RRM2 holoenzyme provides dNTPs for replication and repair in S phase, while RRM1-p53R2 is active in G0/G1 where the consumption of dNTPs is minimal and restricted to mitochondrial DNA replication and repair.

RNR activity is tightly regulated in a cell-cycle dependent manner through multiple mechanisms to coordinate the balance between dNTP synthesis and DNA replication. While levels of RRM1 remain constant throughout the cell cycle, RRM2 levels fluctuate, reaching their highest level in S-phase (7-9). The fluctuation in RRM2 is attributed to transcriptional regulation via the E2F1 transcription factor (10, 11), as well as proteasome-mediated degradation by the anaphase promoting complex/Cdh1 in G1 (12), and by $SCF^{cyclinF}$ in G2 (13). The S-phase specific elevation in RRM2 coincides with a significant increase in RNR activity (9, 14, 15). Thus, RRM2 is considered limiting for RNR activity. Indeed, loss of RRM2 expression results in decreased dNTP levels (16, 17). RNR activity is important to genomic integrity, as well as cell viability, with an increase or imbalance of dNTP pools leading to mutagenesis (18), and decreased dNTP levels leading to impaired DNA replication and repair (5). Previous studies demonstrated that RRM2 transcript and protein levels are increased in cervical cancer lines containing chromosomally integrated HPV16 and HPV18 genomes (19). HPV16 E7 was found to be sufficient to increase RRM2 transcript levels in a manner dependent on E7's interaction with Rb. However, whether RRM2 is increased in lines containing episomal HPV genomes and is important for viral replication has not been examined.

Recent studies have shown that levels of RRM2 can also be regulated through activation of the DNA damage kinase ATR (Ataxia telangiectasia and Rad3-

related) and its downstream effector kinase Chk1 (13, 20-22). The importance of the DNA damage response (DDR) to HPV replication is becoming increasingly clear (23). Previous studies identified a role for the DDR kinase ATM (Ataxia Telangiectasia-Mutated) in productive replication of HPV31 (24), which may facilitate viral DNA synthesis, at least in part, through the recruitment of DNA repair factors to viral replication centers (25, 26). While ATM responds primarily to double-strand DNA breaks, ATR is activated by single-strand DNA generated by DNA damage, as well as by stalled replication forks upon replication stress (27, 28). ATR phosphorylates and activates Chk1, which is then released from chromatin to phosphorylate targets throughout the nucleus (29). Activation of ATR and Chk1 is critical for stabilization of stalled replication forks, and numerous studies suggest that the ATR-Chk1 pathway is essential for cancer cell survival in the face of replication stress (30-32). Recent studies by Bertoli et al demonstrated that the G1/S transcriptional program is rewired in response to replication stress in a Chk1-dependent manner, leading to high-level expression of genes involved in DNA repair, as well as nucleotide synthesis, including RRM2 (20). In addition, several studies have demonstrated that the ATR-Chk1 pathway promotes RRM2 accumulation through stabilization of E2F1, in turn providing dTNPs to prevent DNA damage and cell death (20, 22, 30).

HR HPVs are known to induce replication stress through expression of the E6 and E7 oncogenes (33, 34). E6/E7 expression drives uncontrolled S-phase entry, resulting in a disconnect between replication and supplies required for replication, ultimately leading to DNA damage. Several studies have shown that the ATR-Chk1

pathway is active in HR HPV positive cells (24, 35-37), indicating that replication stress also occurs in the context of a viral infection. ATR-Chk1 activation can be induced independently by E7 (35), as well as the viral helicase E1 (36, 37), and inhibition of Chk1 activity decreases the stability of HPV genomes in undifferentiated cells (38). In addition, the ATR-Chk1 pathway has recently been shown to be required for productive replication of HPV31 (35), though how ATR and Chk1 activity contributes to viral replication was not examined. The activation of ATR and Chk1 in both undifferentiated and differentiating HPV positive cells suggests that replication stress occurs throughout the viral life cycle. Activation of the ATR/Chk1 pathway through HPV-induced replication stress may be required to maintain an environment conducive to the rapid amplification of viral genomes upon differentiation through stabilization of E2F1 and subsequent elevation of RRM2.

In this study, we demonstrate that high-risk HPV positive cells exhibit specific elevation of RRM2 levels, with little to no effect on the large subunit RRM1, or the alternative subunit p53R2. We have found that the increase in RRM2 corresponds with HPV31 positive episomal lines exhibiting higher levels of dNTPs compared to uninfected cells, which importantly, are maintained upon differentiation. Loss of RRM2 expression results in a decrease in viral copy number in undifferentiated cells and a block in productive replication upon differentiation. We have found the levels of RRM2 in HPV31 positive cells are regulated through the ATR-Chk1 DNA damage pathway through maintenance of the E2F1 transcription factor. Overall, our studies suggest that HPV exploits the ATR-Chk1 axis of the DDR to induce E2F1 and RRM2

accumulation upon differentiation, providing a replication-competent environment for completion of the viral life cycle.

MATERIALS AND METHODS

Cell Culture Human foreskin keratinocytes (HFKs) were collected from neonatal foreskin tissue as described previously (39) and were maintained in Dermalife keratinocyte growth media (KGM; Lifeline). HPV31 positive CIN612 9E cells were grown in E-media supplemented with 5ng/mL mouse epidermal growth factor (BD Biosciences) and co-cultured mitomycin C treated J2 3T3 fibroblasts, as described previously (40). Generation and maintenance of HFKs retrovirally transduced with pLXSN-HPV31 E6, pLXSN-31 E7, and pLXSN-31 E6/E7 in combination has been previously described (41). Prior to harvesting DNA, protein or RNA, fibroblast feeder cells were removed from HPV positive cells using Versene (1 mM EDTA in phosphate-buffered saline). 293T cells were grown Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% bovine growth serum (BGS; ThermoFisher Scientific).

Plasmids and Inhibitors The pLXSN-HPV31 E6, -HPV31 E7, and -HPV31 E6/E7 vectors have been previously described (42). The pBR322 plasmid containing the wild-type HPV31 genome was previously described (42, 43). The p1203 PML2d HPV16 plasmid containing the wild-type HPV16 genome was obtained from

Addgene (plasmid no. 10869). VE-821 was obtained from Selekchem, and UCN-01 was obtained from EMD Millipore.

Generation of HPV16- and HPV31-Positive HFKs HFKs stably maintaining HPV16 or HPV31 episomes were created as previously described (40). Briefly, HPV16 and HPV31 genomes were excised from the plasmid backbones using BamH1 and HindIII, respectively (New England Biolabs) and re-ligated using T4 DNA ligase (Life Technologies). Primary HFKs were transfected with 1ug of the re-ligated genomes and 1ug PSV2-Neo using Fugene 6 according to manufactures instructions (Promega). Stable cell lines were generated through eight days of G418 selection (Sigma), and surviving populations were pooled and expanded for analysis.

Induction of Keratinocyte Differentiation High calcium medium was used to induce epithelial differentiation as previously described (44). Sub-confluent cells were harvested as T0, and the remaining plates of cells were serum starved in basal keratinocyte growth medium (KGM; Lonza) with supplements for 16 h. Cells were then incubated in keratinocyte basal medium (KBM; Lonza) without supplements but with 1.8 mM $CaCl^2$ (Sigma). Cells were allowed to differentiate for 24, 48, 72, or 96 h after addition of high calcium medium. DNA, RNA and protein were harvested at each time point, and viral genome amplification was measured by Southern blotting

for each experiment to ensure activation of the productive phase of the viral life cycle.

Production of Lentivirus Lentivirus was produced as previously described (45). Plasmids encoding shRNAs for RRM2 (TRCN0000038962 and TRCN0000038963) were obtained from the UNC Lentiviral Core (UNC-Chapel Hill), and a scramble non-target control shRNA in the pLKO background was obtained from Open Biosystems. Each shRNA plasmid (5ug) was co-transfected with 3.37ug Gag-Pol-Tet-Rev plasmid DNA and 1.66ug vesicular stomatitis virus G (VSV-G) plasmid DNA into 293T cells using polyethylenimine (PEI) to generate lentivirus particles. Supernatants containing lentivirus were harvested 72 h post-transfection, sterile filtered, and stored at -80°C until used. CIN612 9E cells were transduced with 5mL viral supernatant plus 4.8ug/mL hexadimethrine bromide (polybrene; Sigma-Aldrich) for three days prior to harvesting or differentiation in high calcium medium.

Western Blot Analysis Whole cell lysates were harvested in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with Complete Mini and PhosSTOP tablets (Roche). Total protein levels were determined via Bio-Rad protein assay. Equal protein amounts were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The following primary antibodies were used: RRM2, RRM1, p53R2, GAPDH, Involucrin (Santa Cruz); Chk1 (Abcam); phospho-Chk1 Ser345,

E2F1 (Cell Signaling Technologies). Secondary antibodies used were: HRP conjugated anti-goat (Santa Cruz), HRP conjugated anti-rabbit (Cell Signaling Technologies), and HRP conjugated anti-mouse (GE Life Sciences). Clarity Western enhanced chemiluminescence (ECL) blotting substrate (Bio-Rad) was used to detect antibody binding.

Southern Blot Analysis DNA isolation and Southern blotting were performed as previously described (46). Briefly, cells were harvested in DNA lysis buffer consisting of 400mM NaCl, 10mM Tis pH 7.5 and 10mM EDTA. Cells were lysed by the addition of 30uL 20% SDS and subsequently treated with 15ul of 10mg/mL proteinase K overnight at 37˚C. DNA was extracted by phenol chloroform extraction, followed by ethanol precipitation in the presence of sodium acetate. DNA was then digested with BamHI (New England Biolabs) (which does not cut the genome), or HindIII (New England Biolabs) (which cuts the genome once). DNAs were resolved on a 0.8% agarose gel for 15 h at 40 V, and 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.

Measurement of Intracellular dNTPs HFK, HFK-31 and CIN612 cells were seeded in duplicate for each time point at 5 x 10^5 in 10cm dishes. 72hr following seeding, cells were either harvested as a undifferentiated sample (T0) for dNTP

analysis or exposed to high calcium medium for 72hr to induce differentiation. At the indicated time points, cells were lysed in ice cold 65% methanol, and vigorously vortexed for two minutes. Extracts were then incubated at 95 °C for three minutes, chilled for one minute on ice, then centrifuged for three minutes at 14,000 RPM. Supernatants were collected and dried in a speed vacuum. Samples were processed for the HIV-1 reverse transcriptase-based single nucleotide incorporation assay as previously described (47).

Measurement of Ribonucleotide Synthesis Ribonucleotide synthesis was measured as previously described (48). Briefly, HFK and CIN612 cells were seeded in triplicate at 3 x 10^5 cells/well, and 1.5 x 10^5 cells/well in 6 well plates, respectively. Cells were cultured in E-media in the presence of mitomycin C-treated J2 3T3 fibroblasts. Forty-eight hours post-seeding, cells (undifferentiated sample, T0) were labeled with 5.0 µCi/mL D -(U-^{14}C)-glucose in serum-free DMEM containing 200 µM glucose, without pyruvate, for two hours at 37°C. Cells were similarly labeled at 72 hours post-differentiation in high calcium medium. Total RNA was harvested from a separate sample in RNA STAT-60 and extracted according to the manufacturer's instructions (Tel-Test). ^{14}C incorporation was measured by scintillation counting in Ecoscint fluid (National Diagnostics) on a PerkinElmer Tri-Carb 2810 TR. The level of ^{14}C incorporation was normalized to total RNA present in the unlabeled control sample.

Quantitative Reverse Transcription PCR (RT-PCR) Total RNA was isolated from HFKs, HFKs stably expressing HPV31 E6, E7, or E6/E7, as well as CIN612 cells using RNA STAT 60 (Tel-Test), followed by the removal of contaminating DNA via treatment with RQ1 DNAse 1 according to manufacturer instructions (Promega). One microgram of RNA was reverse transcribed using iScript reverse transcription kit (Biorad). Fifty nanograms of cDNA was then analyzed in triplicate reactions using quantitative PCR with 375 nM primers and iTaq Universal SYBR Green Supermix (Biorad) in a total reaction volume of 10ul. Reactions were carried out on an ABI QuantStudio 6 Flex thermal cycler with a thermal profile of 10 min denaturation at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, then 72°C for 30 sec. Data was analyzed using version 1.0 of the QuantStudio 6 and 7 Flex software. The gene-specific primer sequences utilized were as follows: RRM2 (Forward 5'-CTGGCTCAAGAAACGAGGACTG-3'; Reverse 5'-CTCTCCTCCGATGGTTTGTGTAC-3'), RRM1 (Forward 5'-AAAGGAAGAGCAGCGTGCCAGA-3'; Reverse 5'-ACCTCATCCAGACCAGGACAC-3'), E2F1 (Forward 5'-ATGTTTTCCTGTGCCCTGAG-3'; Reverse 5'-ATCTGTGGTGAGGGATGAGG-3'), GAPDH (Forward 3'- CTGTTGCTGTAGCCAAATTCGT -5'; Reverse 3'-ACCCACTCCACCTTTGAC -5'). Relative transcript amounts were calculated using $2^{-\Delta\Delta CT}$ with GAPDH as the reference gene and normalized to uninfected HFK or pLXSN-vector control samples.

RESULTS

RRM2 levels are increased in HPV episomal lines. Previous studies demonstrated that RRM2 protein levels are increased in cervical cancer cells containing integrated viral genomes (19). To determine if RRM2 levels are also increased in lines harboring episomal copies of HPV, we compared the level of RRM2 protein in uninfected human foreskin keratinocytes (HFKs) to HPV31 positive CIN162 cells, which are derived from a CIN1 cervical lesion. We also examined RRM2 protein levels in HPV positive lines generated in the laboratory through transfection of HFKs with HPV31 (HFK-31) and HPV16 (HFK-16) genomes, followed by selection in neomycin. As shown in Figure 3.1A, RRM2 protein levels were substantially higher in CIN612 cells, as well as HFK-31 and HFK-16 cells compared to uninfected HFKs. In contrast, levels of RRM1, as well as the alternative R2 subunit p53R2 were similar between the HFKs and HPV positive lines. To determine if elevated levels of RRM2 were maintained during the productive phase of the viral life cycle, we induced differentiation through growth in high calcium medium, which is commonly used to activate the productive phase of the viral life cycle. As shown in Figure 3.1B, we found that RRM2 levels remained elevated in CIN612 cells upon differentiation compared to uninfected HFKs, which in contrast, sharply declined. Interestingly, while RRM1 levels were again maintained at similar between HFKs and CIN612 cells upon differentiation, p53R2 levels consistently decreased in CIN612 cells (Figure 3.1B). These results suggest that HPV specifically elevates levels of the RRM2 RNR subunit.

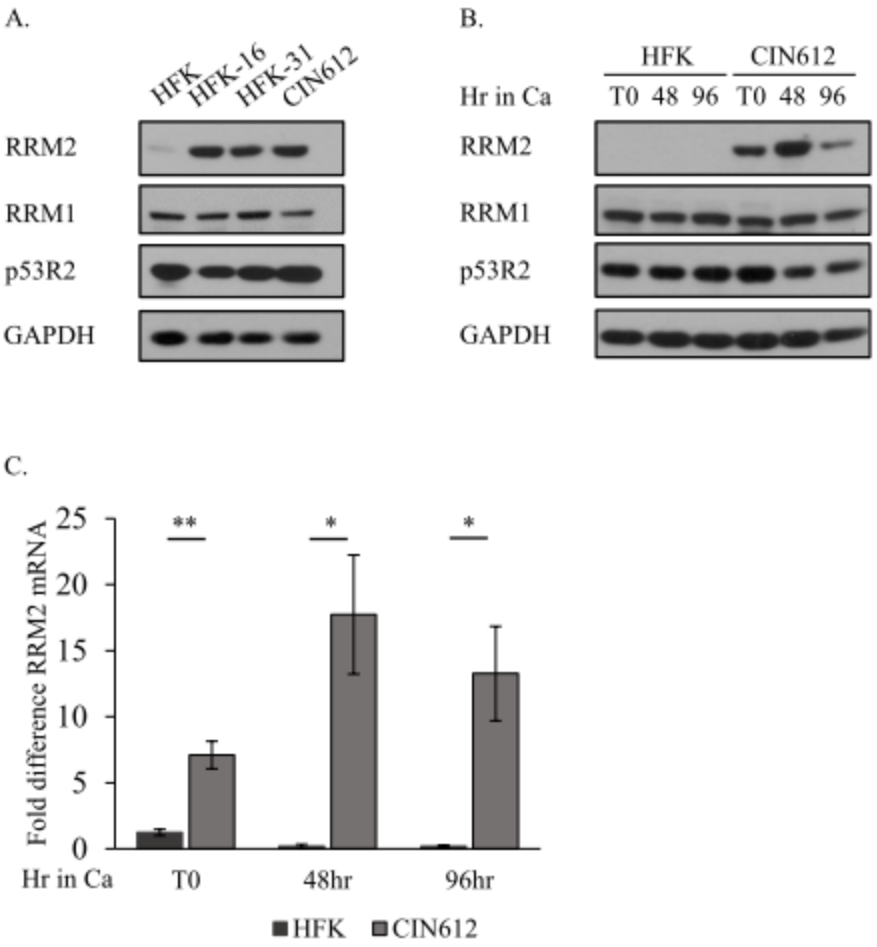

Figure 3.1. RRM2 protein and transcript levels are increased in HPV positive cells. (A) Whole cell lysates harvested from undifferentiated human foreskin keratinocytes (HFKs), HFKs stably maintaining HPV16 (HFK-16) or HPV31 (HFK-31) genomes, and HPV31 positive CIN612 cells were immunoblotted with antibodies to RRM2, RRM1, and p53R2. GAPDH served as a loading control. (B) Whole cell lysates were harvested from undifferentiated (T0) HFKs and CIN612 cells, as well as after differentiation in high calcium medium for 48 and 96hr. Immunoblotting was performed using antibodies to RRM2, RRM1, and p53R2, with GAPDH serving as a loading control. Ca=Calcium. (C) RNA was extracted from HFKs and CIN612 cells

prior to (T0) and post-differentiation for 48 and 96hr in high calcium medium. Quantitative RT PCR was performed using RRM2-specific primers. Expression levels were calculated using GAPDH as the reference gene and are shown relative to the uninfected HFKs (T0), which is set at 1. Fold change was calculated using the 2-ΔΔCT method. Values represent averages from three independent experiments +/- standard error of the means. *p≤ .05, **p≤ .01.

Since RRM2 levels are regulated in part at the level of transcription, we next wanted to determine if the increased RRM2 protein levels in HPV positive cells correlated with increased mRNA levels. As shown in Figure 3.1C, similar to the results observed by Western blot analysis, RRM2 transcript levels were significantly increased in CIN612 cells relative to HFKs prior to differentiation, as well as upon differentiation in high calcium medium. In contrast, RRM2 message levels decreased in HFKs upon differentiation (Figure 3.1C). These results suggest that HPV31 increases RRM2 protein, at least in part, at the level of transcription.

dNTP pools are elevated in HPV31-positive cells throughout the viral life cycle. Levels of RRM2 are thought to regulate the activity of the RNR complex, with higher RRM2 correlating with increased reduction of ribonucleotides to deoxyribonucleotides (dNTPs) (5). To determine if the increased RRM2 levels observed in HPV31 positive cells coincided with elevated nucleotides, we measured dNTP pools in HFKs, CIN612 cells, as well as HFK-31 cells. As shown in Figure 3.2A**,** intracellular pools of dATP, dCTP and dTTP were all markedly increased in

undifferentiated CIN612 cells, as well as HFK-31 cells compared to the matched uninfected HFKs. dGTP levels were also increased, but to a lesser extent. Furthermore, all four dNTP pools were maintained at elevated levels in HPV31 positive cells upon differentiation in high calcium medium (Figure 3.2A). In contrast, dNTP pools were reduced to undetectable levels in HFKs upon differentiation (Figure 3.2A), mirroring the rapid decline in RRM2 protein levels (Figure 3.1B). These results suggest that HPV increases dNTP pools through elevated RRM2 levels and RNR activity.

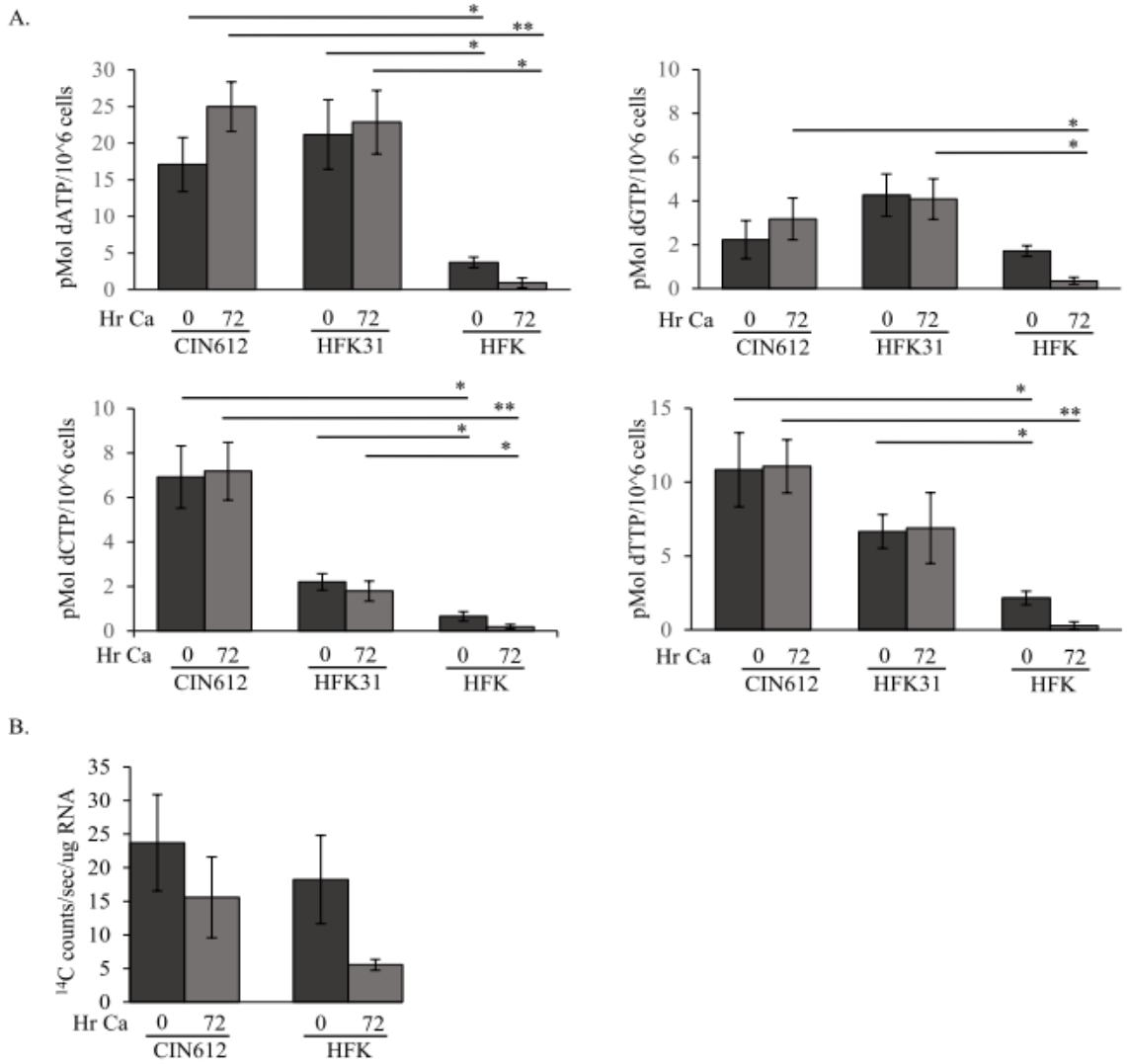

Figure 3.2. HPV31 positive cells exhibit elevated dNTP levels throughout the viral life cycle. (A) Individual dNTP pools were measured in HFKs, HFK-31 cells, as well as CIN612 cells prior to differentiation (T0) and after 72hr differentiation in high calcium medium using a HIV-1 reverse transcriptase-based dTNP assay. Values represent the averages of three separate experiments +/- standard error of the mean. *p≤ .05, **p≤ .01. (B) Total nucleotide synthesis was examined in undifferentiated (T0), as well as differentiated (72hr in high calcium medium) HFK and CIN612 cells by measuring the incorporation of D-(U-^{14}C)-glucose into RNA. Total RNA was extracted and ^{14}C incorporation was determined by measuring counts per minute using a scintillation counter. Counts per minute were then normalized to total RNA for each sample. Values represent the averages of three separate experiments +/- standard error of the mean. Ca=calcium.

We next wanted to determine if the increased dNTP pools observed in HPV positive cells were accompanied by an elevation in ribonucleotide precursors, which are synthesized through the pentose phosphate pathway (PPP) (49). For this, we examined the incorporation of D-(U-^{14}C)-glucose into total RNA as a measure of nucleotide biosynthesis, as described previously (48). Interestingly, as shown in Figure 3.2B, despite higher dNTP pools, undifferentiated CIN612 cells exhibited a similar level of ribonucleotides to that of HFKs, suggesting that nucleotide biosynthesis is not increased by HPV. In contrast, upon differentiation, ribonucleotide levels were maintained in CIN612 cells, while they decreased in HFKs (Figure 3.2B). While this suggests that HPV may activate the PPP upon

differentiation to increase ribonucleotide precursors, we cannot rule out an increased ribonucleotide pool also exists in undifferentiated CIN612 cells, but is rapidly reduced by the RNR complex to yield elevated dNTP pools.

RRM2 is necessary for HPV replication. We have found that both RRM2 and dNTP levels are increased in HPV positive cells prior to and during cell differentiation. These results suggest that HPV may elevate RRM2 levels to drive increased RNR activity to provide dNTPs for viral replication. To determine if RRM2 is required for viral replication, we utilized RRM2-specific small hairpin RNAs (shRNA) and examined the effect of transiently knocking down RRM2 levels on viral replication in both undifferentiated and differentiating CIN612 cells. As shown in Figure 3.3, transient knockdown of RRM2 in CIN612 cells by two different shRNAs resulted in a significant decrease in HPV genome copy number in undifferentiated cells. In addition, decreased expression of RRM2 significantly affected the ability of viral genomes to amplify upon differentiation. Importantly, expression of the differentiation specific marker Involucrin verified that RRM2 knockdown did not prevent epithelial differentiation (Figure 3.3). These results suggest that the increased levels of RRM2 observed in HPV positive cells play an important role throughout the viral life cycle, potentially by providing an adequate supply of dNTPs for viral replication.

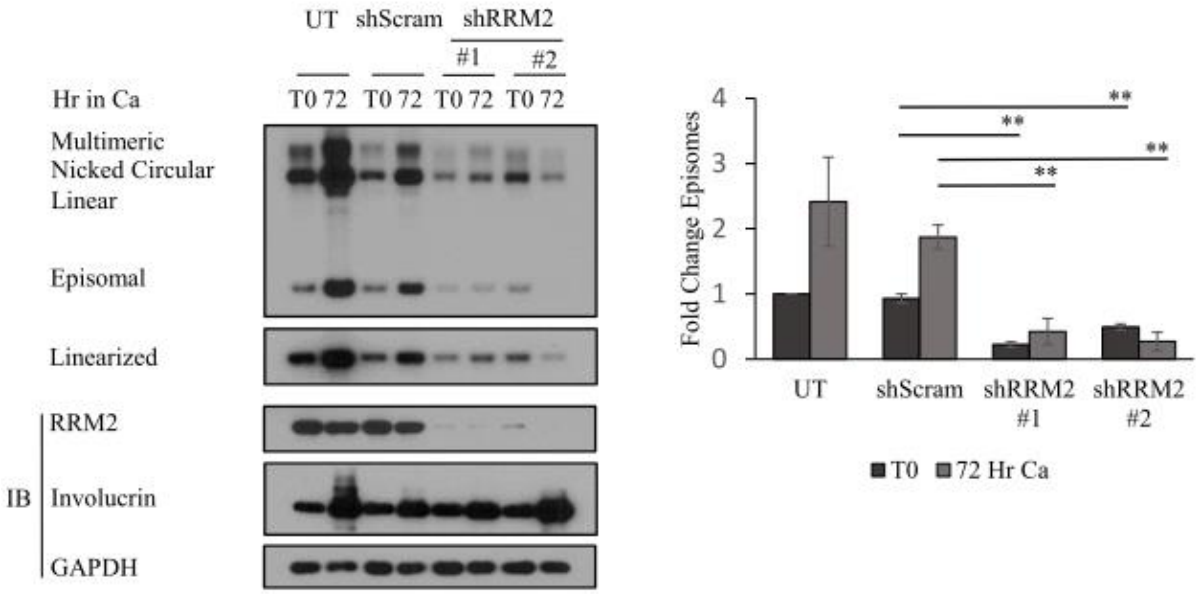

Figure 3.3. RRM2 is necessary for HPV31 replication. CIN612 cells were left untreated (UT) or transiently transduced with either a scramble control shRNA (shScram) or one of two RRM2 shRNAs (shRRM2) for 72hrs. At this time, DNA and protein were either harvested as a T0 (undifferentiated) sample, or cells were exposed to high calcium medium to induce differentiation for 72hr. DNA harvested at each time point was analyzed by Southern blot analysis using the HPV31 genome as a probe. Whole cell lysates harvested at the indicated times points were analyzed by immunoblotting to demonstrate cellular differentiation (Involucrin), as well as levels of RRM2 in shRNA-transduced cells. GAPDH was used as a loading control. Fold change in episome copy number was determined by performing densitometry of episomal bands from three independent experiments using ImageJ software. Shown are the fold changes normalized to episome copy number relative to T0 untreated (UT) CIN612 cells, which is set at 1. Data are +/- standard error of the means. **p≤ .01. IB=Immunoblot. Ca=calcium.

RRM2 is increased in HPV31 positive cells in an E7-dependent manner.

A previous study demonstrated that expression of HPV16 E7 alone is sufficient to increase RRM2 protein levels (19). To determine if HPV31 E7 is also capable of increasing RRM2 levels, we stably expressed HPV31 E6 or E7 alone, as well as E6 and E7 (E6/E7) in combination in HFKs using a retroviral vector. As shown in Figure 3.4A, while HPV31 E6 expression resulted in a slight increase in RRM2 protein levels compared to HFKs, expression of E7 resulted in a marked increase in RRM2 levels that was also maintained in cells expressing E6/E7 in combination. In contrast, minimal effect was observed on the levels of RRM1. While E7 expression alone did result in a slight increase in p53R2 (Figure 3.4A), likely reflecting E7's ability to increase levels of p53 (50), the increase was not maintained in cells expressing E6/E7 in combination, correlating with E6's ability to target p53 for degradation (51). Similar results were observed in a previous study (52). To determine if the E7-dependent increase in RRM2 protein occurred at the level of transcription, we measured RRM2, as well as RRM1 mRNA levels. As shown in Figure 3.4B, levels of RRM2 transcripts were significantly increased (~4-fold) in HFKs expressing E7 alone, as well as E6/E7 in combination compared to control HFKs. Expression of E6 alone resulted in an ~1.5-fold increase in RRM2 transcript levels (Figure 3.4B), mirroring the minor increase in RRM2 protein levels observed. Although we did not observe a detectable effect of E7 or E6/E7 expression on the protein levels of RRM1, both E7 and E6/E7 expression resulted in an ~2-fold increase in RRM1 transcript levels compared to HFKs (Figure 3.4B). This discrepancy between protein and RNA levels is likely due to the extremely long half-

life of RRM1 (~15 hours) (7, 14). Overall, these results suggest that RRM2 is upregulated primarily by HPV31 E7 and that increased transcription contributes to the elevated RRM2 protein levels observed in HPV31 positive cells.

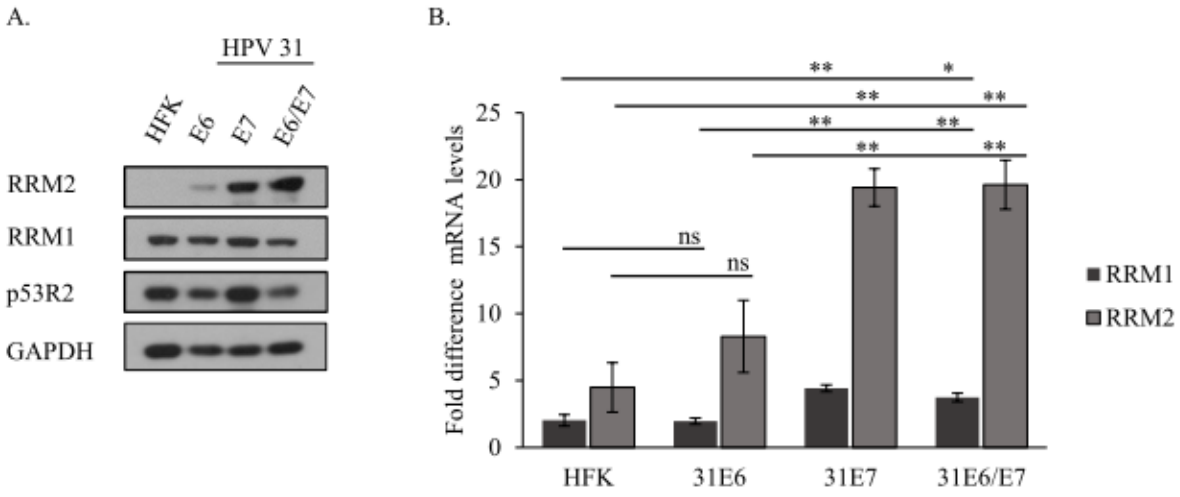

Figure 3.4. HPV31 E7 expression is sufficient to increase RRM2 levels. (A) Whole cell lysates harvested from undifferentiated HFKs and HFKs stably expressing HPV31 E6, E7 or E6/E7 in combination were probed with antibodies to RRM2, RRM1 and p53R2, with GAPDH serving as a loading control. (B) RNA was extracted from HFKs and HFKs stably expressing HPV31 E6, E7 or E6/E7, and quantitative RT PCR was performed to using primers specific to RRM1 and RRM2. Expression levels are shown relative to uninfected HFKs and were calculated using GAPDH as the reference gene. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Values represent averages from four independent experiments across two different HFK backgrounds. Data are +/- standard error of the means. *p≤ .05, **p≤ .01. ns=not significant p>.05.

E2F1 is required for increased RRM2 expression in HPV31 positive cells.

Previous studies demonstrated that RRM2 expression is regulated in a manner dependent on the E2F1 transcription factor (22). E7 is well known for its ability to target Rb and related pocket proteins p130 and p107 for degradation, resulting in deregulation of E2F transcription factors and entry into S-phase (53). As shown in Figure 3.5A, both HPV31 E7-, as well as E6/E7-expressing HFKs exhibited markedly increased levels of E2F1 protein compared to control HFKs, corresponding with the increased levels of RRM2 in these cells (Figure 3.4A). E6-expressing cells exhibited a modest increase in E2F1 protein levels (Figure 3.5A), mirroring the slight increase in RRM2 protein observed (Figure 3.4A). As shown in Figure 3.5B, CIN612 cells also exhibited greatly increased E2F1 protein levels compared to HFKs, and the high level of E2F1 was maintained upon differentiation in high calcium medium. Interestingly, we observed that CIN612 cells, but not uninfected HFKs, consistently exhibited a peak in E2F1 that corresponded with an accumulation of RRM2 at early times post-differentiation (Figure 3.1B, Figure 3.5B). In contrast, this increase was not observed for RRM1 or p53R2.

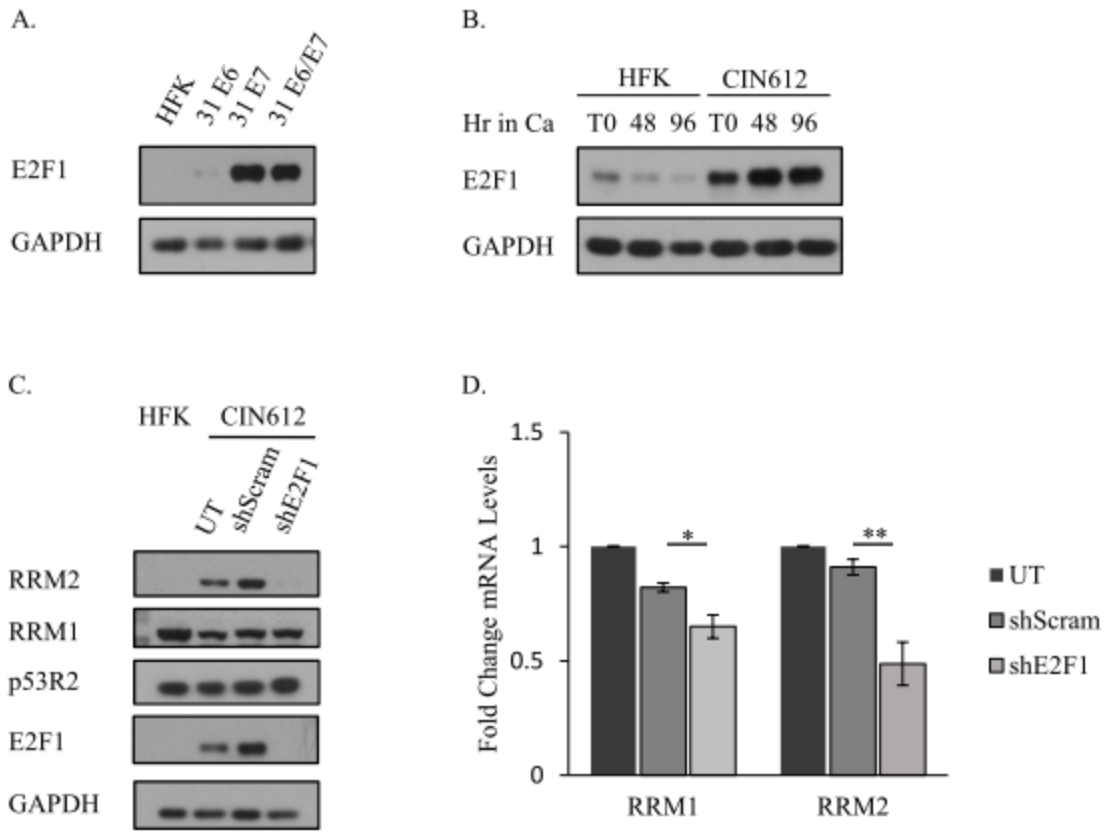

Figure 3.5. E2F1 is required for the increased levels of RRM2 in HPV31 positive cells. (A) Whole cell lysates were harvested from HFKs, as well as HFKs stably expressing HPV31 E6, E7 or E6/E7 in combination and were probed with an antibody to E2F1, with GAPDH serving as a loading control. (B) Whole cell lysates were harvested from undifferentiated (T0) HFKs and CIN612 cells, as well as after 48 and 96hr differentiation in high calcium medium. Immunoblotting was performed using antibodies to E2F1, with GAPDH serving as a loading control. (C) Whole cell lysates were harvested from HFKs, untreated (UT) CIN612 cells, and CIN612 cells transiently transduced with a scramble control shRNA (shScram), or an E2F1 shRNA (shE2F1) for 72hrs. Immunoblotting was performed using antibodies to RRM2, RRM1, p53R2 and E2F1, with GAPDH serving as a loading control. (D) Quantitative RT-PCR was

performed to measure RRM2 mRNA levels in untreated (UT) CIN612 cells, as well as CIN612 cells transiently transduced with a scramble control shRNA (shScram), or an E2F1 shRNA (shE2F1). Expression levels were calculated using GAPDH as the reference gene and are shown relative to the untreated (UT) CIN612 cells, which is set to 1. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Values represent averages from three independent experiments, +/- standard error of the means. *p≤ .05, **p≤ .01.

To determine if the increase in RRM2 in HPV31 positive cells was E2F1-dependent, we examined the effect of E2F1-specific small hairpin RNAs (shRNA) on RRM2 transcript and protein levels in CIN612 cells. As shown in Figure 3.5C, we observed that E2F1 knockdown resulted in a substantial decrease in RRM2 protein levels, with no detectable affect on RRM1 or p53R2. In addition, we found that RRM2 transcript levels were significantly decreased upon E2F1 knockdown (Figure 3.5D), suggesting that E2F1 regulates RRM2 at the transcriptional level in HPV31 positive cells. While we did not observe an effect on RRM1 protein levels upon E2F1 knockdown, we did observe a slight, but significant decrease in RRM1 mRNA levels (Figure 3.5D), which is not surprising as *RRM1* is reported to be an E2F1 target gene (54). Again, the discrepancy observed between RRM1 protein and RNA levels upon E2F1 knockdown likely reflects the long protein half-life of RRM1 (7, 14).

The increase in RRM2 expression in HPV31 positive cells is dependent on the ATR/Chk1 pathway. Increasing evidence supports a link between replication stress, activation of the ATR-Chk1-E2F1 pathway and the accumulation of RRM2. Increased RRM2 expression is thought to prevent DNA damage and maintain cell viability in response to replication stress by providing dNTPs for replication (22, 30, 55). The ATR-Chk1 pathway is constitutively active in HPV positive cells (24, 35), suggesting that viral infection induces replication stress. Recent studies demonstrated that E7 alone is sufficient to induce Chk1 activation (35). These studies raise the possibility that HPV induces RRM2 accumulation through Chk1's ability to increase E2F1 levels. As shown in Figure 3.6A, similar to the results of Hong et al (35), we have found that Chk1 is activated (phosphorylated) to a higher extent in HPV31 positive CIN612 cells compared to HFKs, and that the levels of phosphorylated Chk1 remain elevated upon differentiation. In order to determine if HPV31 increases RRM2 levels through Chk1 activation, we used a chemical inhibitor of Chk1 activity, UCN-01. Previous studies utilizing this inhibitor demonstrated a link between Chk1 activation and RRM2 accumulation in response to replication stress upon S-phase entry (22). In addition, recent studies demonstrated that UNC-01 inhibition of Chk1 activity blocks productive viral replication of HPV31 (35), and we have confirmed those results here (Figure 3.6B).

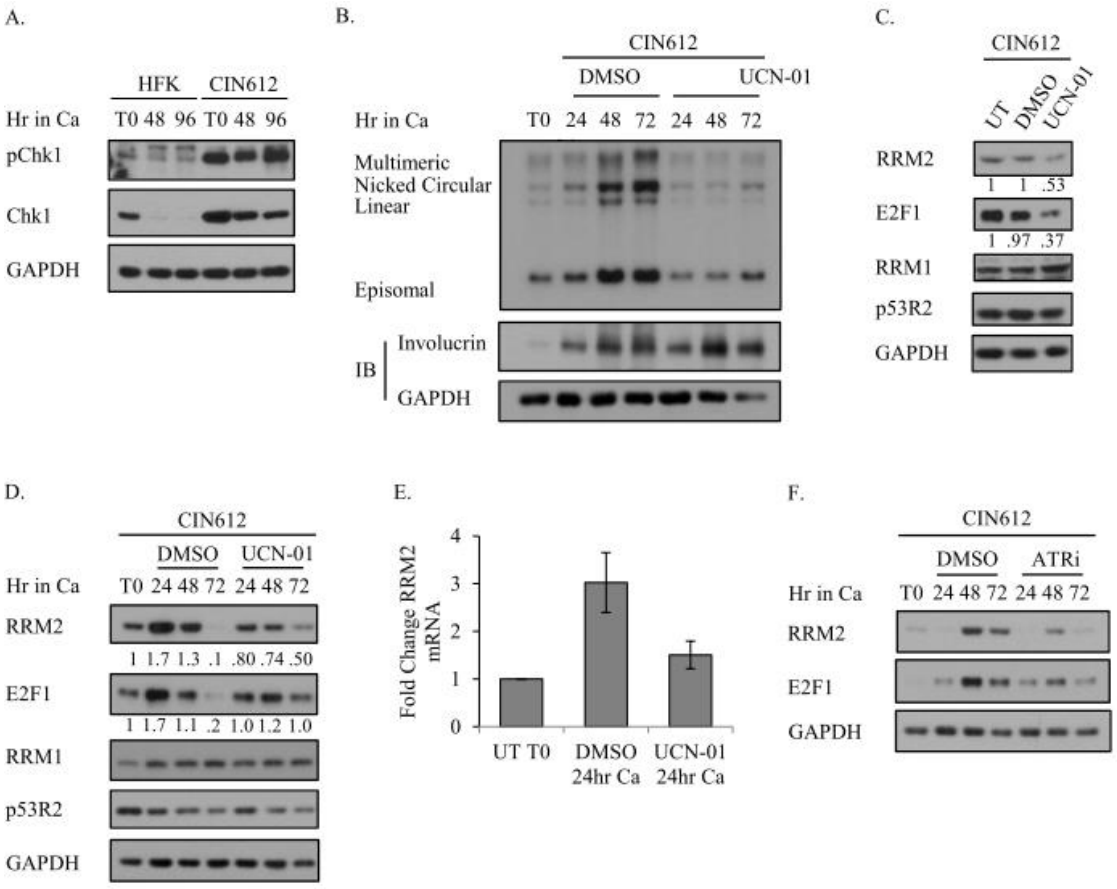

Figure 3.6. HPV31 increases RRM2 levels in a Chk1-dependent manner. (A) Whole cell lysates were harvested from undifferentiated (T0) HFKs and CIN612 cells, as well as after 48 and 96hr differentiation in high calcium medium. Immunoblotting was performed using antibodies to phosphorylated Chk1 (Ser345) and total Chk1, with GAPDH serving as a loading control. (B) DNA and protein were harvested from CIN612 cells at T0 (undifferentiated), as well as after 24, 48, and 72hr differentiation in high calcium media containing either DMSO or 300nM of the Chk1 inhibitor UCN-01. Southern blot analysis was performed using the HPV31 genome as a probe. Immunoblotting was performed using antibodies to Involucrin, with GAPDH serving as a control. (C) Whole cell lysates were harvested from undifferentiated CIN612 cells

that were left untreated (UT) or treated with DMSO or 300nM of the Chk1 inhibitor UCN-01 for 24 hours. Western blot analysis was performed using antibodies to RRM2, E2F1, RRM1 and p53R2, with GAPDH serving as a loading control. Protein levels were quantified by densitometry using ImageJ software and were normalized to the GAPDH loading control. Shown are the fold differences relative to the UT CIN612 cells, which is set to 1. (D) Whole cell lysates harvested from undifferentiated CIN612 cells (T0), as well as CIN612 cells differentiated for 24, 48, and 72hr in high calcium medium containing either DMSO or 300nM UCN-01 were examined by immunoblotting using antibodies to RRM2, E2F1, RRM1, and p53R2, with GAPDH serving as a control. Protein levels were quantified by densitometry using ImageJ software and were normalized to the GAPDH loading control. Shown are the fold differences relative to the T0 CIN612 sample, which is set to 1. (E) RNA harvested from undifferentiated (T0) CIN612 cells, as well as CIN612 cells harvested at 24hr post-differentiation in high calcium in the presence of DMSO or 300nM UNC-01 was analyzed by quantitative RT PCR using primers specific to RRM2. Expression levels were calculated using GAPDH as the reference gene and are shown relative to the UT CIN612 cells, which is set to 1. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Values represent averages from three independent experiments, +/- standard error of the means. (F) Whole cell lysates were harvested from undifferentiated (T0) CIN612 cells and CIN612 cells differentiated for 24, 48, and 72hr in high calcium medium containing either DMSO or 10uM of the ATR inhibitor VE-821. Immunoblotting was performed using antibodies to RRM2 and E2F1, with GAPDH serving as a control. Ca=calcium.

We first examined the effect of Chk1 inhibition on RRM2 and E2F1 protein levels in undifferentiated CIN612 cells. As shown in Figure 3.6C, we found that inhibition of Chk1 activity prior to differentiation resulted in decrease in both RRM2 (~50%) and E2F1 (~37%) protein levels, while having no effect on RRM1 or p53R2. We next examined whether Chk1 activity is required for the accumulation of RRM2 and E2F1 at early times post-differentiation in HPV31 positive cells. For this, we exposed CIN612 cells to high calcium medium in the presence or absence of UNC-01 for 24, 48 and 72hr (Figure 3.6D). Again, while there was no detectable effect of Chk1 inhibition on protein levels of RRM1 or p53R2, the accumulation of RRM2 was blocked at 24hr post-differentiation, as was E2F1. Concomitant with the reduction in E2F1 protein levels upon Chk1 inhibition, RRM2 message levels also decreased by ~2-fold (Figure 3.6E), suggesting that Chk1 increases RRM2 levels in an E2F1-dependent manner.

To further confirm the importance of the ATR-Chk1 signaling pathway in the upregulation of RRM2, we examined the effect of a chemical inhibitor of ATR (VE-821) on the accumulation of RRM2, as well as E2F1 upon differentiation. As shown in Figure 3.6F, similar to the results observed upon Chk1 inhibition, we found that the accumulation of both E2F1 and RRM2 was attenuated upon ATR inhibition. Overall, these results suggest that activation of the ATR/Chk1 pathway in HPV positive cells is required to provide an environment conducive to viral replication by activating E2F1-dependent RRM2 accumulation.

DISCUSSION

RRM2 is considered the rate-limiting component of the RNR enzyme and is required for *de novo* synthesis of dNTPs, along with RRM1 (5). Increased RRM2 levels are associated with greater RNR activity and this is reflected in our finding that along with higher RRM2 levels, HPV positive cells also have higher levels of dNTPs compared to uninfected HFKs. Importantly, high RRM2 and dNTP levels are maintained during the productive phase of the viral life cycle and are likely a necessary resource for the rapid amplification of viral genomes in a differentiating environment. In support of this, we have found that that loss of RRM2 expression leads to a lower viral copy number in undifferentiated cells and a block in productive viral replication upon differentiation. In contrast, while the levels of the alternate R2 subunit p53R2 were similar between undifferentiated HPV positive and HPV negative cells, upon differentiation p53R2 levels decreased in HPV31 positive cells. Though we cannot rule out a role for p53R2 in viral replication, the finding that high levels of RRM2 are maintained, while levels of p53R2 decrease upon differentiation, coupled with the block in productive replication observed upon RRM2 knockdown, argues against this.

The finding that transient knockdown of RRM2 expression leads to a decrease in HPV31 copy number in undifferentiated cells is not entirely surprising given that RRM2 loss is associated decreased dNTP pools (16, 17). Under conditions of limited dNTPs, synthesis of viral DNA is likely to be outcompeted by the host for access to cellular substrates required for replication. Previous studies

with Epstein Barr virus yielded similar results in that inhibition of RRM2 function upon treatment with hydroxyurea, as well as use of RRM2 siRNAs both led to a loss of viral episomes from infected cells (56, 57). Under normal conditions in undifferentiated cells, the small size of the HPV genome, coupled with a low viral copy number, likely does not provide much of a drain on cellular resources, allowing the virus to replicate once per cell cycle along with cellular DNA. Upon differentiation, however, productive replication is thought to occur post-cellular DNA synthesis as cells transition from S- to G2-phase (4), resulting in amplification from 50-100 copies per cell to 1000s of copies per cell. It is possible that the preceding synthesis of cellular DNA upon re-entry into the cell cycle may limit cellular substrates, requiring HPV to increase RRM2 levels to provide dNTPs necessary to facilitate amplification of viral genomes.

It is currently unclear whether the elevation in dNTP pools observed in HPV positive cells is also accompanied by increased ribonucleotide precursors synthesized through the pentose phosphate pathway, which branches from glycolysis at the first committed step (49). While we have found that undifferentiated HFK and HPV31 positive cells exhibit similar levels of ribonucleotides, this may simply reflect the increased reduction of ribonucleotides to deoxyribonucleotides as a result of increased RRM2 levels and RNR activity. In contrast, ribonucleotide levels remain elevated in HPV31 positive cells relative to HFKs upon differentiation, corresponding with maintenance of dNTP pools. Therefore, it is possible that HPV alters nucleotide metabolism at multiple levels to increase dNTP pools.

Understanding the effect of HPV on the pentose phosphate pathway throughout the viral life cycle will be an important area of future research.

Our studies indicate that HPV increases levels of RRM2 through activation of the ATR-Chk1-E2F1 DNA damage response (DDR) pathway, with no detectable effect on RRM1 or p53R2. These results mirror recent findings by Ricardo-Lax et al, in which hepatitis B virus was shown to increase dNTP pools in quiescent cells through increased RRM2 levels in a Chk1-E2F1-dependent manner (58). Activation of the ATR-Chk1 pathway is central to the cell's response to replication stress, and several studies have shown that this pathway is constitutively active in HPV positive cells (24, 35). Importantly, recent studies demonstrated that Chk1 activity is required for productive replication of HPV31 (35), a finding we have confirmed in this study. The constitutive activation of ATR and Chk1 indicates that replication stress may be a constant occurrence in HPV-infected cells and present throughout the viral life cycle. We have found that ATR and Chk1 activity is required for the increased levels of E2F1 and RRM2 in undifferentiated HPV31 positive cells, as well as the accumulation of E2F1 and RRM2 at early times post-differentiation. This observation is reminiscent of studies by Buisson et al who demonstrated that S-phase entry induces replication stress due to a large demand on dNTPs at a time when RRM2 levels are still low (30). This in turn activates the ATR-Chk1 pathway, leading to increased levels of RRM2 in an E2F1-dependent manner to provide dNTPs to maintain genomic stability and cell viability (30). Our finding that activation of the ATR-Chk1 pathway is required for elevated RRM2 levels in HPV31 positive cells suggests that HPV-induced cell cycle re-entry upon differentiation elicits an E2F1

transcriptional response through activation of ATR and Chk1 that culminates in increased RRM2 levels, likely providing dNTPs required for productive viral replication. Importantly, this study indicates that ATR/Chk1 activation is required to maintain a replication-competent environment in differentiating HPV positive cells.

The mechanism by which Chk1 leads to accumulation of E2F1, and in turn RRM2, in HPV positive cells is currently unclear, though several possibilities exist. Chk1 has been shown to phosphorylate E2F6, a negative regulator of E2F-responsive genes, upon replication stress, resulting in its removal from E2F-responsive promoters, allowing E2F1 to bind (20). Other studies have shown that E2F1 is phosphorylated in response to activation of the ATR-Chk1 pathway, leading to increased protein stability and transactivation potential, resulting in increased RRM2 levels (22, 30, 59). Future studies will focus on understanding the link between ATR-Chk1 signaling and increased E2F1 levels in HPV positive cells.

Increasing evidence supports the idea that oncogenes induce replication stress, especially those that promote uncontrolled S-phase entry (60-63). In regards to HPV, several studies have demonstrated that expression of HPV16 E7, and to a lesser extent E6, induce ATR/Chk1 activation (34, 35). In addition, studies by Bester et al demonstrated that expression of HPV16 E6/E7 results in perturbed replication, leading to replication stress and DNA damage attributed to E7's ability to target Rb for degradation and promote cell cycle entry through deregulation of E2F transcription factors (33). Interestingly, a previous study suggested that the HPV16 E7-dependent increase in RRM2 transcription occurs in an E2F-dependent manner and requires an intact Rb binding domain (19). In our study, we have found that

HPV31 E7 alone is also sufficient to increase levels of RRM2, suggesting that this may be a conserved feature of HR HPVs. In addition, expression of HPV31 E7 alone increases the levels of E2F1. Taken together, these results suggest that in HPV-infected cells, E7-induced S-phase entry results in replication stress that leads to increased levels of RRM2 in an ATR/Chk1-E2F1-dependent manner, in turn providing an environment conducive to productive viral replication.

While uncoordinated proliferation can lead to insufficient dNTPs that cause replication stress and promote genomic instability, elevated dNTP pools can also be highly detrimental, leading to DNA breaks, mutagenesis and even cell death (64). A recent survey identified RRM2 as being among the top 10% of overexpressed genes in 73 of 168 cancer analyses that involved multiple types of cancer, including cervical cancer (64). High levels of genomic instability are detected in HPV-associated pre-cancerous lesions (65). Our observation that high-risk HPV positive episomal lines exhibit markedly increased levels of RRM2 suggests that genomic instability could potentially arise in pre-cancerous lesions, at least in part, through increased RNR activity and elevated dNTPs. In addition to *de novo* dNTP synthesis, RRM2 overexpression is also associated with increased cellular invasiveness, angiogenesis, and proliferation in human cancer cells. Recent studies demonstrated that RRM2 overexpression in cervical cancer cells leads to production of reactive oxygen species (ROS) that enhances angiogenesis through HIF-1 alpha and VEGF production (19). The expression of angiogenic factors and increased microvessel density occur very early in the development of HPV-induced pre-cancerous lesions and cervical cancers (66, 67). Understanding if RRM2 overexpression is linked to

increased mutagenesis, as well as angiogenesis in HPV-induced pre-cancerous lesions will provide important insight into the contribution of RRM2 in promoting carcinogenesis of HPV-associated lesions.

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CHAPTER 4: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

GENERAL SUMMARY

In this dissertation I investigated HPV and host keratinocyte interaction, specifically focusing on how HPV engages the host DNA damage response in order to replicate the viral genome. In doing this I investigated viral interaction with two of the major cellular pathways involved in the sensing and repair of DNA damage: the ATM and ATR DNA damage repair pathways. Previous to my research activation of both of these pathways had been shown to be necessary for productive viral replication, but specific contributions of individual proteins and mechanism of activity remained to be investigated (1-3). My research identified specific proteins involved in these pathways that are necessary for productive viral replication, and proposed mechanisms through which they could be contributing to viral replication. I investigated the role of Nbs1 and MRN complex activity in HPV productive replication and how the ATR-Chk1 DNA damage response is involved in maintaining an environment conducive for productive viral replication upon host cell differentiation. Better understanding how HPV engages host DNA damage pathways in order to replicate its genome is important for the development of new and curative antiviral therapies.

In the first chapter of this dissertation I outlined the ATM and ATR DNA damage response pathways. In addition to explaining how they are used to respond to cellular DNA damage I detailed how HPV engages these pathways in order to

replicate its genome. In chapter two I expanded upon what was previously known about the involvement of the ATM DDR in HPV productive replication. I discovered that Nbs1 is necessary for productive HPV viral replication upon host cell differentiation. Specifically, I found that HPV requires Nbs1 and MRN complex activity to recruit homologous repair (HR) factors to viral replication centers, which may be why Nbs1 is required for HPV productive replication. Chapter three deals with the ATR DNA damage response and deoxyribonucleotide triphosphate (dNTP) supply. Previous research has shown that RRM2, an important rate limiting protein involved in dNTP synthesis is upregulated in HPV positive cells (4). More recently Hong et. al. discovered that ATR and Chk1 activity are necessary for HPV productive viral replication, however the mechanism by which it is required was not investigated (3). In my research we observed elevated dNTP pools in HPV positive cells both pre and post differentiation and a ATR-Chk1 dependent elevation of RRM2 levels in these same cells that was necessary for productive viral replication. These results tied together the previously observed elevated RRM2 levels and the requirement for ATR-Chk1 activity to suggest that the ATR pathway is important for providing dNTPs required for productive replication.

Together my research projects discussed in this dissertation further our knowledge of HPV manipulation of host cell factors in order replicate its genome. In this dissertation I have described important pathways utilized by the virus in order to upregulate and recruit factors necessary for viral genome replication. In this final chapter I will integrate these two pathways exploited by HPV in the greater context

of HPV infection and the exciting future research directions raised by these insights into virus/host interaction.

HOMOLOGOUS REPAIR DEPENDENT HPV REPLICATION

HPV is thought to exhibit three major types of viral replication; establishment replication upon initial infection, maintenance replication while the host keratinocyte remains in the basal layer, and productive replication upon differentiation of the host keratinocyte. Since Moody and Lamins first discovered the involvement of the ATM DNA damage pathway in the HPV lifecycle, there has been mounting evidence that ATM and other homologous repair proteins play a role in productive viral replication (1, 2). In my research I set out to elucidate roles for specific proteins in this pathway. The MRN complex composed of Mre11, Rad50, and Nbs1, is known to be upregulated in HPV positive cells, and to localize to sites of HPV genome synthesis. The traditional role of the MRN complex is the recruitment of ATM to the site of DNA double strand breaks. Nbs1 specifically is a well-known downstream effector of ATM and is activated by phosphorylation in an ATM dependent manner. Considering this previous data, we hypothesized that Nbs1 was playing an important role in the HPV lifecycle and viral replication.

The first step in this process was to determine the mechanisms by which Nbs1 and the other MRN components were upregulated in HPV positive cells. Using an established system to express HPV31 E7 in HFKs we determined that E7 expression was sufficient to upregulate levels of all three MRN component proteins. Interestingly, while levels of all three proteins were elevated only Mre11 transcription was significantly increased. This is consistent with Mre11's role as the binding

partner of both Nbs1 and Rad50 and its ability to stabilize both proteins (5, 6). To determine the importance of Nbs1 upregulation and recruitment to HPV DNA centers, I used a validated shRNA to knock down Nbs1 protein levels and observe the effect on HPV replication.

The hypothesis was, since ATM activity was necessary for HPV productive replication and Nbs1 is known to be an important activator of ATM, depletion of Nbs1 would lead to a defect in productive viral replication due to a lack of ATM activation. Interestingly, although productive replication was affected in a manner similar to that seen in ATM deficient cells in previous studies, there was no consistent or significant effect on ATM or Chk2 activation as measured by phosphorylation. This is inconsistent with a model requiring Nbs1 mediated ATM activation to promote productive viral replication. Instead this data suggested both, that an alternative ATM activating pathway must be at work and that Nbs1 must be affecting productive replication outside of ATM activation. Downstream of ATM activation, Nbs1 is known to play a role in both cell cycle control at the intra-S-phase checkpoint and DNA damage induced apoptosis however neither of these pathways were affected by Nbs1 knockdown.

In addition to these functions downstream of ATM activation, previously published data suggested that protein-protein interaction motifs on Nbs1 were important for recruitment of the MRN complex (7, 8) and other HR proteins including Rad51 (9) to nuclear foci. Furthermore, activity of the MRN complex and other proteins recruited by Nbs1 is known to be important for the processing of broken ends of DNA and initiation of HR (10). To see if Nbs1 depletion was affecting these

pathways I knocked down Nbs1 in HPV positive cells and conducted a cytoplasmic-nuclear fractionation. This was compared to fractionations performed on untreated HPV positive cells and normal HFKs. The fractionation showed an enrichment of nuclear MRN in the infected cell compared to the HFK. However, in the absence of Nbs1, levels of nuclear Mre11 and Rad50 decreased and instead these proteins were found localized to the cytoplasm. In addition, through the use of HPV genome specific FISH and immunofluorescence we found that Mre11, Rad50, and Rad51 were recruited to HPV DNA foci in the nucleus both prior to and post differentiation with significant increases in Mre11, Rad50 and Rad51 positive foci observed upon differentiation. Interestingly while Nbs1 depletion abrogated this recruitment of Mre11 and Rad50 at all time points, Rad51 recruitment was only negatively affected upon differentiation. Furthermore, I was able to show that not only was Mre11 recruited to viral replication centers by Nbs1, but using the Mre11 nuclease inhibitor mirin, I was able to show that Mre11 nuclease activity was also necessary for productive viral replication. Nbs1 is thought to recruit Mre11 to broken DNA ends where Mre11 processes these broken ends of DNA by resection. These finding support the current hypothesis that, upon differentiation HPV switches to a HR dependent method for viral DNA synthesis, since Mre11 nuclease activity at the sites of replication may be necessary for the recruitment of further HR factors such as Rad51 (11). In fact, this is further supported by even more recently published research from the Moody lab showing that the HR factors Rad51 and Brca1 are necessary for productive viral replication upon differentiation. These results, in combination with other previously published studies, suggest a model in which HPV

engages ATM, which signals through Nbs1. This Nbs1 activity may be important for recruitment of HR factors to sites of viral DNA synthesis. Otherwise, the lack of amplification occurring in the absence of Nbs1 may be leading to the lack of further HR factors being recruited. Then, upon differentiation this signaling is magnified and the assembled factors facilitate massive viral DNA synthesis through a HR dependent mechanism.

The model of a switch to an ATM and HR dependent mechanism for productive viral genome replication upon differentiation has been growing in strength since Moody and Laimins first found that ATM and Chk2 activity were necessary for productive viral DNA replication but not genome maintenance (1). The work detailed in my second chapter further supports this hypothesis by identifying two further ATM dependent HR proteins required for productive viral replication that have little effect on viral genome maintenance. Further research on this topic has been conducted in our laboratory by Chappell et al (12), showing that Brca1 and Rad51 are also necessary for productive viral replication. This is interesting since Brca1 is known to work in concert with Mre11 in the resection of double strand breaks and then to aid in the recruitment and stabilization of Rad51 at sites of DNA damage. Together with my research this suggests a model in which Nbs1 is bringing Mre11 and Rad51 to sites of viral DNA synthesis upon differentiation and that the interaction of these proteins are necessary for productive replication (Figure 4.1). Conversely it is possible that Rad51 is recruited in an Nbs1 independent manner and Mre11 activity at the site of DNA synthesis is responsible for the accumulation of Rad51. This could be potentially explored by repeating the mirin treatment of HPV positive cells and

observing the localization of Rad51 via FISH/IF as was done in the case of Nbs1 knock down. This hypothesis could be further investigated by expressing either Nbs1 deficient in Mre11 binding or vice versa, to determine if recruitment of Mre11 is an essential activity of Nbs1 necessary for productive viral replication. Finally, it would be interesting to determine if HR itself was essential for HPV replication. Chemical inhibition of DNA-PK auto phosphorylation or overexpression of DNA-PK with mutations in the auto phosphorylation cluster site has been shown to block progression of HR by preventing DNA-PK release from broken DNA ends, preventing end processing and the recruitment of HR factors (13, 14) (reviewed in (15)). It may be interesting to use these systems in HPV positive cells to determine if recruitment of HR factors to sites of HPV replication or productive genome replication was affected.

MODULATION OF ATR DNA DAMAGE PATHWAY TO PROVIDE REPLICATION FACTORS

As detailed in the introduction the HPV lifecycle is tightly regulated and linked to host cell differentiation. In order to avoid detection from the immune system HPV infection in basal keratinocytes exists in a maintenance state replicating its genome once per cell cycle along with the host genome. It is not until the host cell begins differentiate and move upward away from the basal layer and immune surveillance, that productive viral replication takes place (16). However, since differentiating cells have exited the cell cycle, this means that HPV must somehow manipulate the host cell environment in order to provide factors necessary for viral genome amplification to thousands of copies per cell. As previously outlined the virus accomplishes this

through the expression of its early genes, especially E6 and E7 which target p53 and Rb respectively. In addition, a growing body of evidence points to activation of the cellular DNA damage pathways in order for HPV to alter cell cycle regulation. Work covered in the second chapter of this dissertation focused on the ATM DNA damage pathway, a well-studied way in which HPV alters cell cycle regulation and provides a replication competent atmosphere. In addition, recent evidence suggest that HPV may also be targeting the ATR mediated DNA damage pathway in order to allow for productive replication upon host cell differentiation. The third chapter of my dissertation describes one mechanism by which HPV infection may be exploiting the ATR mediated DNA damage pathway in order to provide factors necessary for HPV viral DNA synthesis.

Recently published research has shown that ATR is constitutively activated in HPV positive cells and that this activation is required for successful productive HPV replication upon differentiation, however no mechanism was proposed (3). Also, separate research has shown that the small subunit of RNR, RRM2 is upregulated in HPV positive cervical cancer cells in an E7 dependent manner (4). I therefore set out to examine the importance of both this ATR activation and RRM2 upregulation in the context of HPV infection, especially as it affected HPV viral genome replication. Previous work had only measured RRM2 levels in cervical cancer cells where the viral genome has integrated, RRM2 levels had not been examined in the context of the complete differentiation dependent lifecycle. Since the largest amount of HPV viral genome synthesis occurs during productive replication upon differentiation I verified that RRM2 was upregulated upon differentiation. Upregulation of RRM2

protein levels and gene expression were not only present upon differentiation in HPV positive cells, but were increased compared to HFKs. Importantly since RRM2 is thought to be a major rate limiting component in the synthesis of dNTPs, we verified that levels of all dNTPs were indeed upregulated in HPV positive cells both prior to and upon differentiation. Next, using an RRM2 specific shRNA I determined that RRM2 is necessary for HPV productive replication. Interestingly I also observed this productive replication deficiency when I treated differentiating HPV positive cells with Chk1 inhibitors, recapitulating the results seen by Hong et al. (3). In addition, we saw a significant decrease in both E2F1 and RRM2 protein levels in these cells. The decrease in E2F1 was important because activation of ATR has previously been shown to upregulate RRM2 levels in a Chk1-E2F1 dependent manner. These results, combined with the observation that ATR inhibition and E2F1 knock down in HPV positive cells also decreased RRM2 expression, led me to a model in which HPV is engaging the ATR DNA damage response, either directly or through the creation of replication stress in order to upregulate RRM2 expression to provide dNTPs for viral DNA synthesis in differentiating cells (Fig 4.1). This is important because, while ATR activation in HPV positive cells had been observed for some time, this model suggests a reason why this activation may be helpful to the virus. Also, the model proposes a method by which the virus may be manipulating the host cell to provide one of the most important factors for viral DNA synthesis, dNTPs. Elevated RRM2 levels and dNTP pools being necessary for the replication of an oncovirus is an interesting result. Recent research has shown that elevated RRM2 levels correlate to a poor cancer prognosis (17-20). Also, lower levels of RRM2 and

a subsequent lack of dNTPs is thought to lead to oncogene-induced senescence in cancer cells (21-23).

While this work and the resulting model is an important step forward in understanding the role of ATR and Chk1 signaling through E2F1 to RRM2 in the HPV lifecycle and genome replication some questions remain unanswered. More must be done in order to fully explain the link between ATR-Chk1 signaling, RRM2 upregulation, dNTP supply and productive viral replication. First, while I have shown that both RRM2 and dNTPs are upregulated in HPV positive cells, I have not shown that the increase in dNTPs is a direct result of RRM2 upregulation. I have shown that there is an increase in ribonucleotide pools in HPV positive cells, however it would be useful to determine if RRM2 knockdown in these cells resulted in lower dNTP pools as well as a defect in viral genome replication. This could strengthen the hypothesis that HPV is upregulating RRM2 production in order to supply dNTPs for viral genome synthesis. Also, more could be done to strengthen the argument that the increased RRM2 expression is necessary to provide dNTPs for viral genome replication and that this is indeed regulated by a Chk1-E2F1 interaction. Rescuing productive viral reproduction by suppling deoxy ribonucleosides would be an obvious first step. Also, a rescue of replication in the presence of ATM or Chk1 inhibition using an RRM2 or E2F1 expression vector would strengthen the argument that E2F1 regulation by ATM-CHk1 signaling is important. One final point not addressed in this work that would be interesting to investigate, would be the contribution of the upstream pathways involved in nucleoside production including the pentose phosphate pathway. While we have determined that the steady state pools of

ribonucleotides are similar in undifferentiated HFK and HPV positive cells and only slightly elevated in HPV positive cells upon differentiation, we have not determined if higher levels of ribonucleosides are being produced, but are just as quickly being reduced by the increased RRM2 activity.

DISSERTATION IMPACT

In order to successfully infect its host, reproduce and pass on its genetic material every virus must accomplish two things: avoid or repress immune detection and commandeer necessary cellular machinery in order to express its proteins and replicate its genome. Through its relatively small size and coding capacity and its cell cycle dependent regulation of gene expression and genome replication, HPV does a fairly good job at accomplishing immune evasion. However, these same factors make viral genome replication even more complicated. One of the most challenging problems faced by HPV is how to massively replicate its genome in a cell that has exited the cell cycle. As outlined in this dissertation HPV is known to interact with many host cell signaling pathways in order to push the host cell back into the cell cycle and to freeze the cell in what is thought to be a G2/M arrested state (Fig. 4.1). The question addressed in my dissertation is how in this state, does HPV regulate and recruit host factors necessary for productive viral replication.

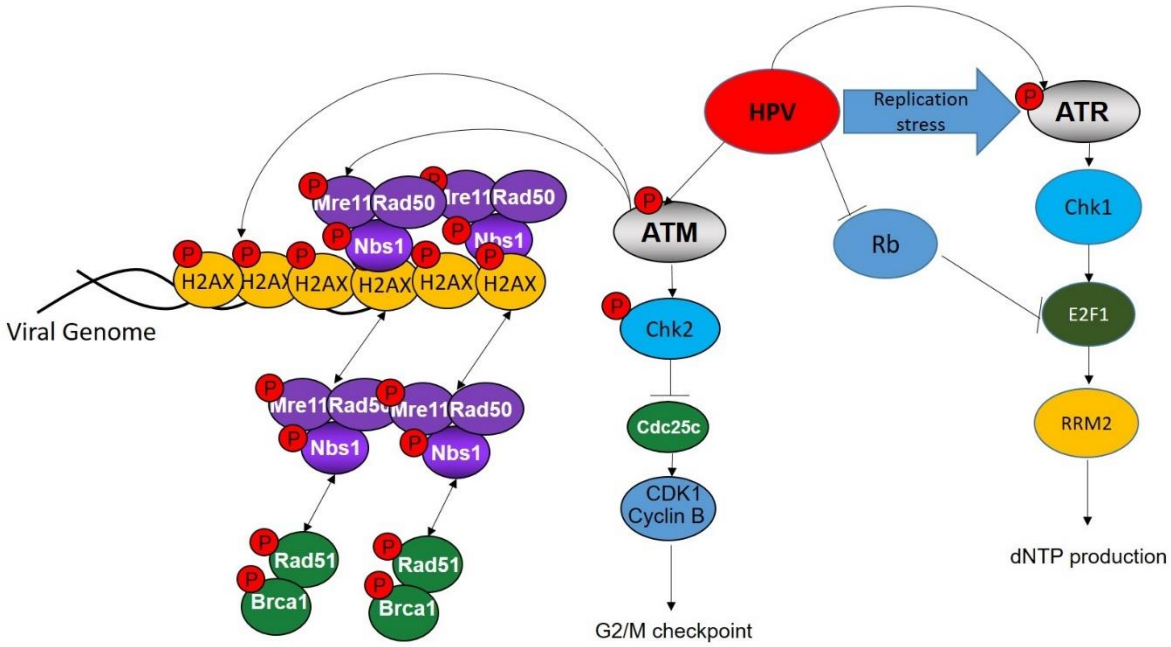

Figure 4.1 HPV E7 engages both the ATM and ATR DDR pathways in order to recruit host factors and promote viral genome amplification.

My work begins to unite the current ATM and HR dependent productive viral replication model with a hypothesis that HPV also exploits ATR signaling, to further activate E2F factors to activate transcription of factors important for viral genome replication (Fig 4.1). Currently, it is believed that upon host cell differentiation, viral genome replication switches from the bidirectional theta replication seen in maintenance phase infection to a different form of DNA synthesis. This separate form of viral genome replication has been shown to require many components of the ATM DDR and HR (Fig 4.2) (1, 12, 24, 25) (reviewed in (11)).

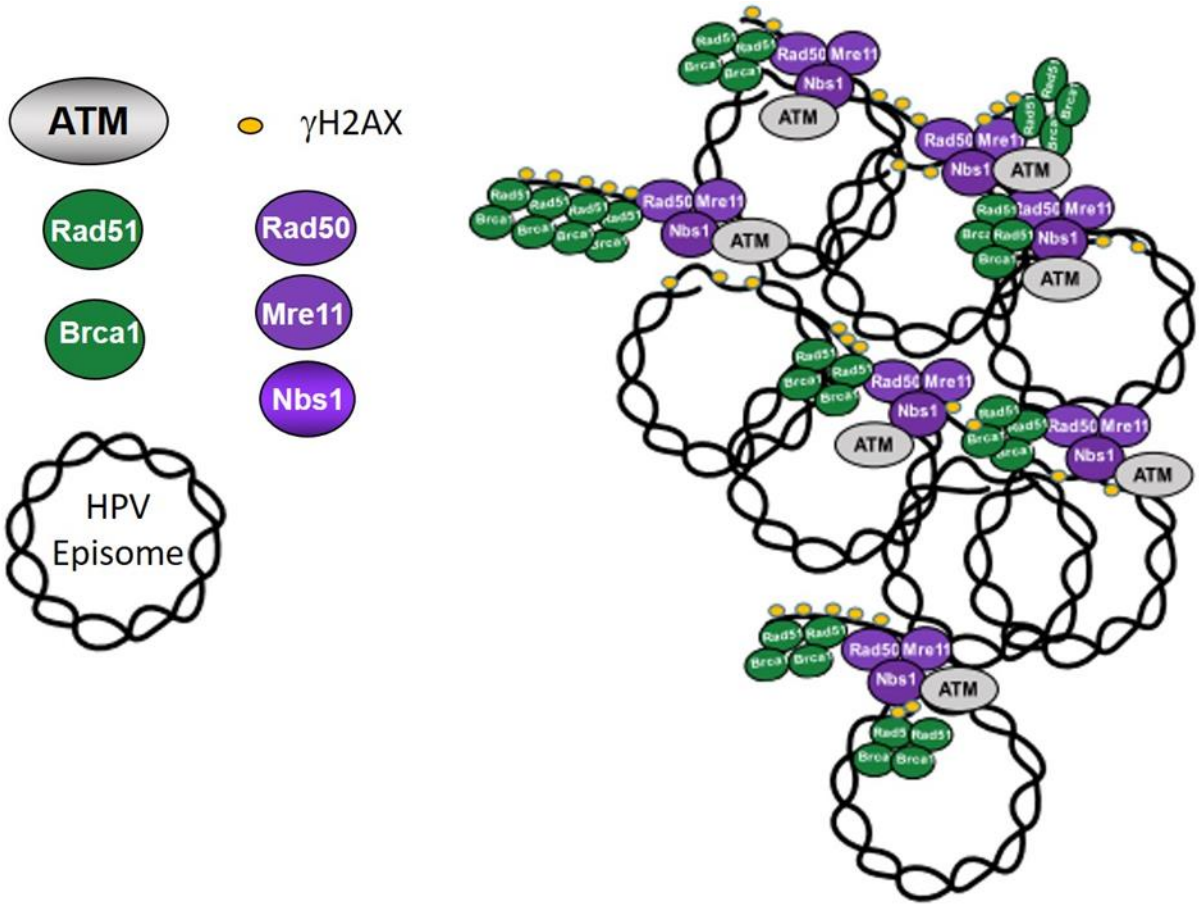

Figure 4.2 DNA HR factors are recruited to HPV replication centers in order to facilitate HR dependent viral genome amplification. Several HR factors have been shown to be recruited to sites of HPV viral genome amplification. These factors have been shown to be necessary for productive viral genome amplification upon host cell differentiation. I propose a model in which these factors are recruited to breakage sites in HPV episomes. Neighboring episomes are then used as a template for synthesis of a new viral episome. In this way, tightly packed HPV episomes serve as templates to prime the synthesis of new episomes in a manner similar to sister chromatids serving as template DNA for HR of double strand breaks in the host genome. This process, happening simultaneously from multiple sites on

the many tightly packed HPV episomes present in replication foci, could result in the massive and rapid amplification of HPV episomes observed during host cell differentiation.

The discovery of Nbs1 involvement in this productive viral replication is important because, not only is it known to interact with ATM in the DDR, but Nbs1 is also recruited to sites of DNA damage and is known, together with the other components of the MRN, to recruit important repair factors to these sites. This role for Nbs1 and the MRN in the DDR is consistent with my observations of the necessity for Nbs1 and Mre11 activity for HPV productive replication. In the context of HPV replication, I found that Nbs1 and the activity of the MRN complex are required, but somehow outside of ATM activation. My research suggests that Nbs1 is important for productive viral replication due at least in part to its ability to recruit other HR factors including Mre11. Overall, productive replication of HPV genomes is a poorly understood process, hopefully further understanding of the recruitment of HR factors to sites of viral genome replication will lead to methods by which we can block this replication and the spread of infection.

While we are still researching the HR factors recruited to sites of viral genome replication through ATM and Nbs1 and how these HR factors impact viral DNA synthesis, it is still important to understand how the necessary dNTP pools for this synthesis are maintained in differentiating cells. Typically, the post mitotic differentiating cell would not provide necessary factors for large scale DNA synthesis. While HPV infection does push the cell back into the cell cycle and is thought to hold the cell in a G2/M arrested state, this is still not an ideal state for

DNA synthesis. However, I have shown that dNTP pools are maintained at a high level in differentiating HPV positive keratinocytes. Furthermore, I have described a pathway by which HPV may be engaging the ATR DDR to activate RRM2 expression in order to ensure the availability of these dNTP pools (Fig 4.1). These discoveries are important because they give weight to new evidence that ATR is required for productive HPV replication. This data suggests that drugs targeting RRM2 may be useful antivirals against HPV infection and replication.

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